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# Pyridoxine Dependent Epilepsy:

# Diagnostics and outcome of the Dutch patients

**Vinus Bok**

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Coverpage 'De Poortwachter', by Annemiki Bok, 2008 Espel

*De ogen van de Poortwachter stralen wilskracht, hoop en liefde uit. Maar ook bezorgdheid en angst. Angst voor de dreigende en het onbekende, wat van boven komt. Dit schilderij symboliseert ook Pyridoxine Dependent Epilepsy; de dreiging van aanvallen uit het brein, de hoop op genezing en een normaal leven. De wilskracht en liefde om door te gaan, op weg naar verbetering van de toekomst voor patiënten met Pyridoxine Dependent Epilepsy en hun families.*

Lay-out by Henk Dinnissen
Printed by Drukwinkel Emmeloord

ISBN no: 978-90-9027682-3
NUR no: 876

Financial support for the publication of this thesis has been kindly provided by

**Stichting René**

máxima medisch centrum

Radboud Universiteit Nijmegen

eurocept pharmaceuticals

# Pyridoxine Dependent Epilepsy:

# Diagnostics and outcome of the Dutch patients

Proefschrift
ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het college van decanen
in het openbaar te verdedigen op dinsdag 10 september 2013
om 12.30 uur precies

door

Levinus Adrianus Bok
geboren op 26 april 1958
in de Noord-Oostelijke Polder

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## Contents


| | | |
|---|---|---|
| **Chapter I.a** | General introduction and aims of the study | 7 |
| **Chapter I.b** | Epidemiology of pyridoxine-dependent seizures in the Netherlands<br>*Arch Diseases of Childhood 2005 Dec; 90(12):1293-1296* | 11 |
| **Biochemical and Molecular Genetic Studies** | | 21 |
| **Chapter II.a** | Pyridoxine dependent seizures in Dutch patients: diagnosis by elevated urinary alpha-aminoadipic semialdehyde levels<br>*Arch Dis Child 2007 Aug; 92(8):687-689* | 23 |
| **Chapter II.b** | The measurement of $\Delta^1$-piperideine-6-carboxylate, the alter ego of $\alpha$-aminoadipic semialdehyde, in Antiquitin deficiency<br>*J Inherit Metab Dis 2012; 35:909-916* | 31 |
| **Chapter II.c** | An intriguing "silent" mutation and a founder effect in *antiquitin* (*ALDH7A1*)<br>*Ann Neurol 2007 Oct; 62(4):414-418* | 43 |
| **Clinical Studies in PDE** | | 53 |
| **Chapter III.a** | The EEG response to pyridoxine-IV neither identifies nor excludes pyridoxine-dependent epilepsy<br>*Epilepsia 2010 Dec; 51(12):2406-2411* | 55 |
| **Chapter III.b** | Antenatal treatment in two Dutch patient with Pyridoxine Dependent Seizures<br>*Eur J Pediatr 2010 Mar; 69(3):297-303* | 67 |
| **Chapter III.c** | Long-term outcome in pyridoxine-dependent epilepsy<br>*Dev Med & Child Neurol 2012 sept; 54(9):849-854* | 79 |
| **Chapter III.d** | Roth spots in Pyridoxine Dependent Epilepsy<br>*BMJ-Case Reports 2011: 17 aug* | 93 |
| **Chapter IV** | General Discussion and Future Perspectives | 97 |
| **Chapter V** | Summary | 109 |
| **Addendum** | Samenvatting in het Nederlands | 114 |
| | Dankwoord | 119 |
| | Curriculum Vitae | 121 |

# Chapter I.a

## General introduction and aims of the study

## General introduction

In 1954, Andrew Hunt and others reported on a child with a severe convulsive disorder who responded to regular administration of pyridoxine (vitamin B6)[1]. The authors preferred the term 'pyridoxine dependency' instead of 'pyridoxine deficiency' and they hypothesized that a metabolic anomaly of pyridoxine metabolism was present in this patient.

Pyridoxine Dependent Epilepsy (PDE) is a very rare disease with a reported incidence of 1:783 000 in Great Britain and of 1:250 000 in the Netherlands[2,3]. In PDE seizures start in utero as 20 - 25% of the mothers perceive abnormal foetal movements during pregnancy, or seizures start at birth or soon after birth. Seizures can start even months after birth. In PDE other non-convulsive clinical symptoms than seizures can be observed[4]. These non-convulsive symptoms indicate that PDE is essentially an encephalopathy with epilepsy as the most dominant symptom.

For decades there was great uncertainty among doctors how to identify or to exclude PDE in patients with therapy resistant seizures. The reasons for this dilemma were several; i.e. the great clinical heterogeneity and the age of presentation of seizures, the variety of clinical symptoms and the rarity of PDE, and because the metabolic defect remained unclear for half a century. Another reason was that in children with seizures with or without PDE additional therapeutic effects of pyridoxine can be seen. This dilemma might explain the use of terms as classical PDE, non-classical PDE, pyridoxine dependent, pyridoxine responsive, pyridoxine deficient, possible PDE, probable PDE and definite PDE which were used in the past.



For many years scientists focused their research in PDE patients on a metabolic defect in the GABA synthesis pathway in which pyridoxine acts as a cofactor. However a metabolic defect in GABA metabolism could never be demonstrated. In 1998 a locus for PDE on chromosome 5q31 was suggested but the disease relating gene could not be identified[5]. Plecko reported elevated plasma and cerebrospinal fluid levels of pipecolic acid in 2000 but the significance of this remained unanswered[6]. In 2006 a British-Dutch consortium (Research leaders Peter Clayton and Cornelis Jakobs) discovered that the metabolic defect of PDE is based on a disorder in lysine metabolism[7]. Furthermore mutations were found in the *ALDH7A1* gene, also known as the antiquitin gene, which is indeed located on chromosome 5q31.

Prior and independently from these findings, the epidemiology of PDE in the Netherlands was reported in 2005 (Jasper Been and Levinus Bok)[3]. This study based on clinical definitions presented 11 PDE patients with a calculated birth incidence of PDE in the Netherlands of 1:252 000 newborns.

In 2006, two Dutch groups, under the leadership of Prof. Dr Cornelis Jakobs, Metabolic Unit, Department of Clinical Chemistry, VU University Medical Centre, Amsterdam and Prof Dr Michèl Willemsen, Department of Pediatric Neurology, Radboud University Nijmegen-Medical Centre, Nijmegen, collaborated on a PDE research project. Since 2006 the number of PDE patients in the Netherlands increased from 11 (based on clinical definition) to 22

patients (metabolic and genetically confirmed). The studies – as described here – aim to investigate epidemiological, biochemical, molecular genetic and clinical aspects of PDE. Many other scientists, doctors and hospitals collaborated in these studies as all patients are from all regions of the Netherlands. We thank all who cooperated in these studies.

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# Chapter I.b



## Epidemiology of pyridoxine-dependent seizures in the Netherlands

Been Jasper Valentijn, Bok Levinus Adrianus, Andriessen Peter, Renier Willy Omer

Arch Diseases of Childhood 2005 Dec; 90(12):1293-1296

## Abstract

**Introduction**: Pyridoxine-dependent epilepsy is a rare cause of seizures in childhood. The diagnosis is made upon clinical criteria, that in many cases are never met. Therefore, epidemiologic data on pyridoxine dependency are scarce.

**Objectives**: To study the epidemiology of pyridoxine dependent epilepsy in the Netherlands, and to determine whether the diagnosis is based upon the appropriate criteria.

**Methods**: Nationwide all departments of paediatrics (n = 113) and of paediatric or neonatal neurology (n = 17) were asked to report cases of pyridoxine-dependent seizures. Birth incidences were calculated using national data on live births from 1991 to 2003.

**Results**: Response was received from 67% of paediatric departments, including all university hospitals and 94% of child neurology departments. Thirteen patients were reported. Four definite (31%), three probable (23%), and four possible cases (31%) were identified. Two cases (15%) did not meet criteria for either of these groups. The birth incidence was 1:396 000 for definite and probable cases and 1:252 000 when possible cases are included.

**Conclusions**: Thus far, epidemiologic data on pyridoxine dependent seizures were only available from the UK and Ireland. A higher incidence was found in the Netherlands, in accordance to earlier suggestions of a regional difference. The study shows that the diagnosis is often made without performance of a formal trial of withdrawal. We want to underline the importance of confirming the diagnosis, concerning the consequences as for individual prognosis, the potential side effects of prolonged pyridoxine substitution, and the possibility of treating the mother in case of future pregnancies.

## Introduction

Pyridoxine-dependent epilepsy is a rare autosomal recessive disorder with a classic presentation of onset of seizures in the first days of life that are intractable to conventional anti-epileptics[1,2]. In pyridoxine dependency, seizures will generally cease several minutes after parenteral administration of pyridoxine (vitamin B6). The diagnosis is established when convulsions recur after withdrawal of pyridoxine (within days to weeks) and cease again after a second trial of pyridoxine[2]. In general, the patient will be free of seizures after institution of pyridoxine maintenance monotherapy. Atypical forms include those with seizures only partly responsive to pyridoxine, referred to as pyridoxine-responsive seizures, and those with late onset of seizures[2].

Few reports have been made of epidemiological data concerning pyridoxine dependency. A regional study in the United Kingdom (UK) published in 1996 by Baxter, reported a point prevalence of definite cases of 1:100 000[3]. When the study was extended to the UK and Ireland in 1999, a point prevalence of definite and probable cases of 1:687 000 and a birth incidence of 1:783 000 were found[4]. These and other observations support the presence of a regional variation of the incidence of pyridoxine-dependent seizures[3-5]. It has been suggested that the incidence as reported by Baxter in 1999 is probably an underestimation of the true prevalence[4-6]. Concerning the low prevalence of pyridoxine-dependent seizures, patients are likely to be underdiagnosed. Moreover, in many cases a formal trial of pyridoxine withdrawal, required for establishment of the diagnosis, is never performed. The need for additional demographic studies of pyridoxine-dependent seizures has been recognised[5], yet up to date none have been performed.



The aims of this study were 1) to study the epidemiology of pyridoxine-dependent seizures in the Netherlands (total population January 1st 2004: 16 258 000); and 2) to determine whether the diagnosis is based upon the appropriate clinical criteria.

## Methods

A questionnaire by letter was sent to all heads of paediatric departments in the Netherlands, whom were asked to report any case of pyridoxine-dependent seizures born between 1980 and 2003. Likewise, all paediatric neurologists and neonatologists of the neonatal neurology working group were individually approached to report cases. Respondents were asked to fill out a questionnaire. The referred data were reported and stored anonymously, for which no ethical approval is necessary in the Netherlands. The criteria of definite, probable, and possible cases of pyridoxine-dependent seizures were applied as published by Baxter in 1999[4]. Birth incidences were calculated over the period of January 1991 to December 2003, using the total number of life births in the Netherlands during this period as adapted from http://statline.cbs.nl.

## Results

Response was obtained from 76 of 113 paediatric departments (67%), including all university hospitals (n = 8) and from 16 of 17 departments of paediatric and/or neonatal neurology (94%). Eighteen notifications of known pyridoxine-dependent cases were received, including five duplicate reports. Each patient was at least reported by one academic specialist or child neurologist. In addition, one patient from our personal experience was included (patient 10 in table I).

Of all patients reported only one was born before 1991. Also, this was one of two patients of whom only sparse clinical data were retrieved. Many respondents mentioned difficulty reporting patients or retrieving clinical data from several years ago. Therefore, we decided to limit the period down to patients born between January 1991 and December 2003, as mentioned previously. Thus, 13 patients were included in total, one of whom has previously been reported (patient 3)[7]. The total number of live births during this period was 2 771 397.

Four patients (31%) met the criteria for definite pyridoxine-dependent seizures. Three probable cases (23%) and four possible cases (31%) were defined. The birth incidence of definite and probable cases was 1:396 000. When possible cases are included, the birth incidence was 1:252 000.



Two patients (15%) did not meet the criteria for either definite, probable, or possible pyridoxine dependency. One patient had seizures responsive to pyridoxine and was initially diagnosed with pyridoxine dependency after a trial of withdrawal had been carried out. Yet one week after the trial, seizures recurred and became unresponsive to pyridoxine. At age 5 months, folinic acid was added to his treatment regimen. Analysis of the cerebrospinal fluid (CSF) showed the presence of the typical marker for folinic acid-responsive seizures. Yet despite extensive anti-epileptic therapy including pyridoxine and folinic acid, the patient still is not fully seizure-free.

The second patient had seizures directly after birth that responded clinically, but not electrophysiologically to pyridoxine. A trial of withdrawal was never performed, and she had never been seizure-free on pyridoxine monotherapy. Interestingly, intrauterine seizures had been present in this patient, while in contrast only one definite case was reported to have had intrauterine seizures.

The characteristics of the reported patients are summarised in table I. Two probable cases each had a sibling with definite pyridoxine dependency (patients 6 and 7). Also, two possible cases were sisters (patients 1 and 5). Only few clinical data on patient 6 were available. Nevertheless, this patient was included since she was a sister of patient 4. She had seizures responding to pyridoxine and has been seizure-free on pyridoxine, however no trial of withdrawal has been performed. Reports of gender and race of the patients and consanguinity of the parents were incomplete.

Signs of fetal distress, e.g. meconium stained amniotic fluid or abnormal cardiotocography,

**Table I.** Characteristics of definite, probable, and possible pyridoxine-dependent patients

| Patient | Birth year | *Definite* | *Probable* | *Possible* | Gender | Intrauterine seizures | Age at 1st seizure | Age at 1st pyridoxine | Initial dose (mg) | Age at trial | Maintenance dose (mg/day) | Development | Other major diagnoses or complications |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 (5) | 1991 | | | X | F | no | 2 days | 9 days | 150 | - | 50 | delayed initially but currently normal | - |
| 2 | 1992 | X | | | ? | no | 4 days | 6 months | 100 | 1.7 years | 100 | delayed; attends special school | - |
| 3 (7) | 1992 | X | | | F | no | < 1 day | 2 months | 25 | 2 months | 60 | normal | - |
| 4 (6) | 1992 | X | | | ? | no | 8 days | 2 months | 100 | 2 months | 100 | mildly affected motor skills | - |
| 5 (1) | 1993 | | | X | F | no | 2 days | 2 days | 50 | - | 50 | psychomotor delay; attends special school | PPHN,birth asphyxia |
| 6 (4) | 1994 | | X | | F | ? | ? | ? | ? | - | 100 | normal | - |
| 7 (3) | 1995 | | X | | M | no | 8 days | 8 days | 60 | - | 60 | lost to follow-up | - |
| 8 | 1998 | | | X | M | no | < 1 day | 3 days | 50 | - | 50 | delayed | hydrocephalus, cerebral palsy |
| 9 | 2001 | X | | | ? | no | 3 days | 8 months | 50 | 8 months | 50 | psychomotor delay; poor language skills | - |
| 10 | 2003 | | X | | F | yes | < 1 day | 2 days | 50 | 2 days | 50 | psychomotor delay | - |
| 11 | 2003 | | | X | M | no | 1 day | 2 weeks | ? | - | 100 | normal | - |

('number') = patient number of sibling; PPHN = persistent pulmonary hypertension of the newborn

had been present in two definite and two possible cases (patients 3 and 10, 5 and 8 respectively). Patient 5 experienced an episode of persistent pulmonary hypertension and suffered perinatal asphyxia with signs of cerebral ischemia on ultrasonography. Convulsions in this patient were initially ascribed to hypoxic-ischemic encephalopathy. Yet, since her sister had previously experienced neonatal seizures responsive to pyridoxine, pyridoxine was administered. On this, seizures ceased, and electroencephalography normalised. Both girls are currently seizure free on pyridoxine monotherapy, yet in neither one a trial of withdrawal has been performed.

## Discussion

Thus far, epidemiological data on pyridoxine-dependent seizures were only available from the UK and Ireland. As reported in this paper, a higher incidence of possible, probable, and definite pyridoxine-dependent cases was found in the Netherlands. This is in accordance with previous reports of a regional difference in the prevalence of pyridoxine-dependent seizures. The relative proportion of definite, probable, and possible cases in our study was 36%, 27%, and 36%, respectively. These percentages are very similar to those reported by Baxter: 39%, 25%, and 36%, respectively[4]. Therefore, regional differences in diagnostic skills are unlikely to account for the different incidences between the two studies. A genetic factor is likely to play an important role, since there were three sibling cases in our small study.



Moreover, our study confirms that the diagnosis is often being made without application of the appropriate clinical criteria. Of all thirteen patients reported, eight (62%) had been diagnosed with pyridoxine-dependent seizures without the formal trial of withdrawal having been carried out. Parents may be reluctant to a trial of pyridoxine withdrawal, because they fear it will cause harm to the child[1]. On the other hand, we believe that in many cases physicians may never consider a trial of withdrawal due to insufficient knowledge of this rare disorder. A standardised therapeutic approach to neonatal seizures has previously been suggested to increase the awareness and improve the recognition of classical pyridoxine-dependent seizures[5]. In the Netherlands, a treatment protocol including a pyridoxine trial is increasingly used to address neonatal seizures in a standardised manner. We believe that this will indeed increase the recognition of pyridoxine-dependent seizures and support an appropriate establishment of the diagnosis. Such an approach should advice administration of parenteral pyridoxine 50 - 100 mg as a test dose in neonates with seizures intractable to conventional anti-epileptics. Clinicians should be aware of possible cardiorespiratory depressive effects of a first pyridoxine administration. Recently, there have been promising reports on pipecolic acid being a possible diagnostic marker of pyridoxine-dependent seizures[8,9]. It has even been suggested that measurement of pipecolic acid may be sufficiently sensitive to replace a trial of withdrawal[9]. Therefore, we think measurement of pipecolic acid in plasma and/or CSF should be included in such a protocol as well. Additional data on this issue need to be collected in order to clearly establish the value of pipecolic acid in the diagnosis of pyridoxine-dependency. Unfortunately, no data are available on pipecolic acid measurements in the patients reported here.

Confirmation of the diagnosis is reassuring for the patient and his or her family and is of great importance. It may have consequences for genetic counselling and facilitates a more precise prognosis. Furthermore, confirmation of the diagnosis in a young child may warrant pyridoxine administration to the mother in case of a future pregnancy, which in turn may prevent developmental delay in a subsequent pyridoxine-dependent child[10]. On the other hand, a trial of withdrawal may identify patients who have wrongfully been diagnosed with pyridoxine dependency. Previous data have shown that a large minority of patients treated as having pyridoxine-dependent seizures in fact remain permanently seizure-free after pyridoxine withdrawal[4]. This is important since needless maintenance treatment with high doses of pyridoxine may cause a serious, although largely reversible, dorsal root gangliopathy[11]. No hard data are available as to what would be the proper timing for a trial of withdrawal. Probably it is better not to perform a trial shortly after the initiation of pyridoxine supplementation. Baxter has advocated the trial to be carried out before school entry, i.e. around the age of four years[1].

All patients reported in this study had relatively early onset of seizures, while the initial presentation of pyridoxine-dependent seizures may occur up to the age of 9 months. This may be due to both underreporting and underrecognition of late-onset pyridoxine dependency. Early-onset seizures in pyridoxine dependency are known to be associated with a poorer outcome, especially when treatment initiation is delayed[12]. In our study however, only half of all patients were reported to have a substantial degree of developmental delay. Also there was no clear correlation between the age of treatment and outcome. Earlier reports on this issue have been conflicting. Baxter and Aicardi have suggested that earlier pyridoxine treatment is beneficial, and this has been confirmed by Plecko et al., whereas Haenggeli et al. found no correlation between the time of treatment and outcome[2,8,12]. Conclusions are to be drawn with caution however, since the number of patients is relatively small, and comparative performance tests have not been performed.



The data presented in this study are based upon the cooperation of the clinicians addressed. A methodological limitation of the study is related to the retrospective character of the study. Therefore, cases of pyridoxine-dependent patients may be missed due to lack of response, leading to an underestimation of the actual incidence. Overall the response was limited, yet virtually all university hospitals and neurological departments replied. Since each patient was reported by at least one of the latter, we believe an incomplete report of patients is not very likely. Unfortunately, our study period had to be narrowed due to the inability of many respondents to retrieve clinical data from several years back. Theoretically this may have caused a reduced report of patients as well, although the relatively even birth year distribution of the reported patients over time does not support this. On the other hand, the high number of sibling cases may have led to an overestimation of the incidence of pyridoxine-dependent epilepsy.

In conclusion, we think our data are indicative of a reliable number of patients born between 1991 and 2003 who are believed to be pyridoxine-dependent in the Netherlands. Pyridoxine dependency seems to be more prevalent in the Netherlands than in the UK and Ireland, supporting earlier suggestions of a regional variation in the demographics of pyridoxine dependency. However our results confirm that still the diagnosis is regularly made without

application of the appropriate clinical criteria. On the other hand, many patients may never have been recognised and may continue to be unsuccessfully treated with conventional anti-epileptics. A better knowledge of the disease entity and the clinical criteria needed for establishment of the diagnosis will contribute to heightened awareness and more adequate management of potentially pyridoxine-dependent seizures. A standardised treatment protocol for neonatal seizures including a pyridoxine trial may be of additional value, at least as long as pyridoxine-dependent epilepsy remains a clinical diagnosis.

**Acknowledgements**
We would like to thank all replying clinicians for their cooperation, in particular the following clinicians for kindly providing the patient data: Dr. W. Baerts, Isala Klinieken, Zwolle; F.M.C. van Berkestijn, Universitair Medisch Centrum, Utrecht; A.N. Bosschaart, Forum Educatief, Utrecht; I.F.M. de Coo, Erasmus Medisch Centrum, Rotterdam; G.A.P.T. Hurkx, Elkerliek Ziekenhuis, Helmond; R. Kohl, Het Spittaal, Zutphen; Dr. L.A.E.M. Laan, Leids Universitair Medisch Centrum, Leiden; Dr. K.D. Liem, Universitair Medisch Centrum St. Radboud, Nijmegen; J. Nicolai, Academisch Ziekenhuis Maastricht; R.F.H.M. Tummers, Medisch Spectrum Twente, Enschede; Dr. A. van der Wagen, Streekziekenhuis Midden-Twente, Hengelo.

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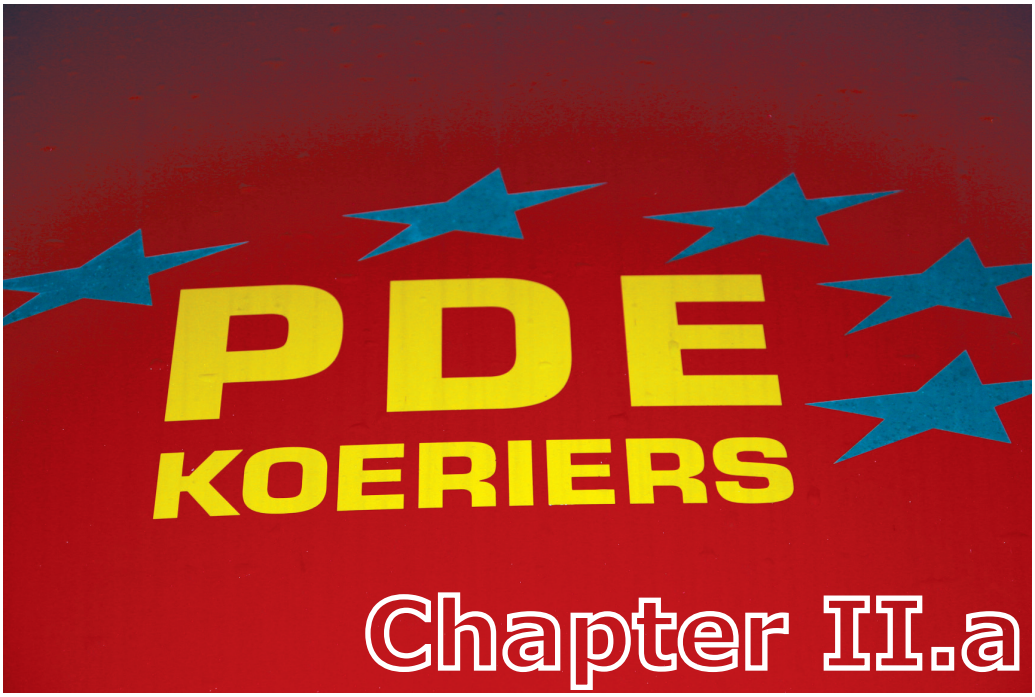
# Biochemical and Molecular Genetic Studies






# Pyridoxine-Dependent Seizures in Dutch patients: diagnosis by elevated urinary alpha-aminoadipic semialdehyde levels

Bok Levinus A., Struys Eduard, Willemsen Michel A.A.P., Been Jasper V., Jakobs Cornelis

Arch Dis Child 2007 Aug; 92(8):687-689

## Abstract

**Background**: Pyridoxine Dependent Seizures (PDS) is a rare, autosomal recessively inherited disorder. Recently α-aminoadipic semialdehyde (α-AASA) dehydrogenase deficiency was identified as a major cause of PDS, which causes accumulation of both α-AASA and pipecolic acid (PA) in body fluids.

**Methods**: We studied urinary and plasma α-AASA and PA levels in 12 Dutch clinically diagnosed PDS patients.

**Results**: α-AASA was elevated in both urine and plasma in 10 patients. In these patients plasma PA levels were also elevated but urinary PA levels were normal.

**Discussion**: In all patients with clinically definite PDS, and in most patients with probable or possible PDS, the clinical diagnosis of PDS could be confirmed at the metabolite level. Non-invasive, urinary screening for α-AASA accumulation provides a reliable tool to diagnose PDS and can save these patients from the classical and potentially dangerous pyridoxine withdrawal test to prove PDS.


### What is already known on this topic

- PDS is a rare disease caused by alpha-aminoadipic semialdehyde (α-AASA) dehydrogenase deficiency.
- Epidemilogical data on PDS are rare and are based on clinical criteria for PDS.



### What this study adds

- Non-invasive, urinary screening for α-AASA accumulation provides a reliable tool to diagnose PDS.
- The birth incidence on metabolic confirmed PDS of this nationwide dutch study is estimated to be at least 1:276 000.

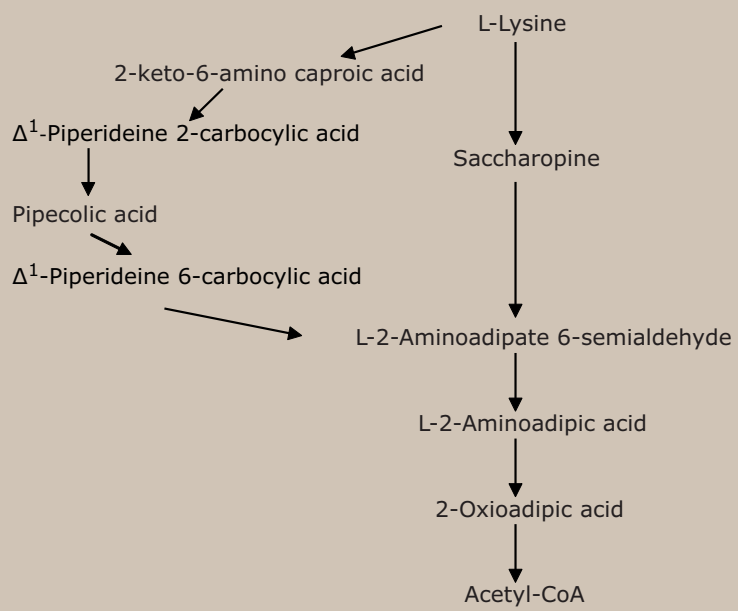
## Introduction

Pyridoxine dependent seizures (PDS) is a rare, autosomal recessively inherited disorder usually presenting very shortly after birth and in some cases in the womb. For 50 years PDS has been a clinical and biochemical conundrum which has puzzled physicians and scientists[1]. Plecko et al and Willemsen et al observed isolated pipecolic acid (PA) elevations in plasma and cerebrospinal fluid in PDS patients, yet the biochemical relation with pyridoxine metabolism remained unclear[2-4]. Recently, α-aminoadipic semialdehyde (α-AASA) dehydrogenase deficiency due to pathogenic mutations in the *ALDH7A1* gene, was shown to be a major cause of PDS[5]. In mammals, the essential amino acid L-lysine is degraded via PA into the intermediate α-AASA, which is subsequently oxidized to L-2-aminoadipic acid, a reaction catalyzed by the enzyme α-AASA dehydrogenase (EC 1.2.1.31, also named antiquitin)(Fig. I). In PDS patients the lack of α-AASA dehydrogenase leads to an accumulation of α-AASA and PA in body fluids. α-AASA is in spontaneous reversible equilibrium with piperideine-6-carboxylate (P6C) in the cytosol. Accumulated P6C irreversibly reacts with active pyridoxine, ie, pyridoxal-5-phosphate (P5P), by forming a Knoevenagel condensation product (Fig. II). This irreversible reaction causes a secondary deficit of P5P in affected children, which subsequently leads to epileptic seizures. Restoration of the P5P pool can easily be achieved by oral pyridoxine supplementation, and resolves the seizures.

Recently we reported on the epidemiology and clinical features of PDS in the Netherlands in this journal[6]. In that paper the classical clinical criteria according to Baxter were used to establish the diagnosis of PDS. We therefore reevaluated that series of PDS patients by measuring their levels of α-AASA and PA in urine and plasma.



**Figure I.** Metabolic pathway of L-Lysine



## Methods

We re-evaluated all children (n = 11) with a diagnosis of definite, probable or possible PDS from a recently described Dutch cohort of 13 patients[6]. These patients and their parents were invited to visit our hospital and were informed about the novel insights into the pathophysiology of PDS. All except one patient (patient 11) underwent further diagnostic work-up by laboratory investigations of urine and blood. Furthermore, we were able to include a recently born sibling of patient 10 in the present study (patient 12).

α-AASA in urine and plasma was measured by liquid chromatography-tandem mass spectrometry as previously published[5]. Quantitative determination of PA in urine and plasma was performed by stable isotope dilution gas chromatography-mass spectrometry[7].

## Results

Urine and/or blood samples were obtained from 11 patients. The results of α-AASA and PA measurements are listed in table I. α-AASA was elevated in urine and plasma of 10 patients. PA in plasma was elevated in all patients with elevated (plasma and urine) α-AASA, while urinary PA concentrations were normal in all patients.

**Table I.**

| **Patient** | **Sibling** | **Birth Year** | **PDS (Baxter criteria)** | **AASA urine** (mmol/ mol cr.) | **AASA plasma** (µmol/L) | **PA urine** (mmol/ mol cr.) | **PA plasma** (µmol/L) | **PDS** (**metabolic confirmed**) |
|---|---|---|---|---|---|---|---|---|
| **1** | 5 | 1991 | possible | 16 | 8.0 | 0.11 | 6.5 | **Y** |
| **2** | | 1992 | definite | 4.7 | 0.9 | 0.27 | 5.8 | **Y** |
| **3** | 7 | 1992 | definite | 29 | 5.7 | 0.00 | 4.6 | **Y** |
| **4** | 6 | 1992 | definite | 4.0 | 1.1 | 0.02 | 7.0 | **Y** |
| **5** | 1 | 1993 | possible | 24 | 5.0 | 0.11 | 5.0 | **Y** |
| **6** | 4 | 1994 | probable | 0.2 | < 0.2 | 0.02 | 2.2 | **N** |
| **7** | 3 | 1995 | probable | 20 | 5.8 | 0.01 | 5.1 | **Y** |
| **8** | | 1998 | possible | 12 | 2.4 | 0.07 | 5.5 | **Y** |
| **9** | | 2001 | definite | 9.6 | 0.8 | 2.0 | 22 | **Y** |
| **10** | 12 | 2003 | probable | 39 | 6.1 | 0.08 | 7.8 | **Y** |
| **11** | | 2003 | possible | N.A. | N.A. | N.A. | N.A. | **N** |
| **12** | 10 | 2004 | | 75 | 5.2 | 1.37 | 11 | **Y** |

- N.A. = not available; Y = yes; N = not
- Control α-AASA concentrations are < 0.2 µmol/l for plasma, and < 1 mmol/mol creatinine for urine.
- Control values for PA in urine are 0.55 - 24.1 mmol/mol creatinine (< ½ year of age) and 0.01 - 1.54 mmol/ mol creatinine (> ½ year of age).
- For PA in plasma, control values are 3.75 – 10.8 µmol/(< 1 week of age), and 0.7 - 2.46 µmol/l (> 1 week of age).

## Discussion

In this study, in all patients with a definite diagnosis of PDS according to the criteria published by Baxter[8], α-AASA dehydrogenase deficiency could be proven at the metabolite level by demonstrating elevated concentrations of α-AASA (plasma and urine) and PA (plasma). The diagnosis was also confirmed in two out of three patients with probable PDS and in three out of four patients with possible PDS.

The diagnosis of probable PDS could not be confirmed in one patient (patient 6). She is a younger sister of a girl with a definite clinical diagnosis of PDS and metabolic confirmed diagnosis of α-AASA dehydrogenase deficiency (patient 4). She had subtle neonatal seizures with only minimal epileptic discharges on a 24-hour EEG, which responded to 100 mg of pyridoxine given intravenously. She is performing well at school. Her normal development makes a diagnosis of PDS even more unlikely since most PDS patients suffer from - at least a mild – encephalopathy with learning difficulties[5]. However, the nature of the neonatal seizure-like period remains unexplained. We advised a trial period of pyridoxine withdrawal but could not convince the parents to stop pyridoxine treatment. DNA analysis of patients included in our study are pending.

In patient 11, originally diagnosed with possible PDS, pyridoxine was recently withdrawn without recurrence of seizures. We consider PDS a very unlikely diagnosis in this patient because of the above observation and the fact that the child develops well. The parents did not want to cooperate with further metabolic investigations.



In our first report[6] we reported two patients (patients 12 and 13 in that paper) who did not meet the criteria for either definite, probable of possible PDS. In both patients we have now demonstrated normal α-AASA concentrations in plasma and urine, as would be expected (data not shown).

This report is the first nationwide population-based study on metabolic confirmed PDS. Our results show that at least 10 children with PDS were born in the Netherlands between January 1991 and December 2004. As 2 764 697 children were born in the Netherlands during this time (adapted from http://statline.cbs.nl), the birth incidence of biochemical proven PDS in the Netherlands is at least 1: 276 000 children. This study further shows that most patients, namely 9 out of 11 (82%), were diagnosed correctly using the criteria proposed by Baxter. Thus, in circumstances where metabolic examination of α-AASA and/or PA is not possible, applying the clinical criteria proposed by Baxter seems a reliable method to establish a diagnosis of PDS.

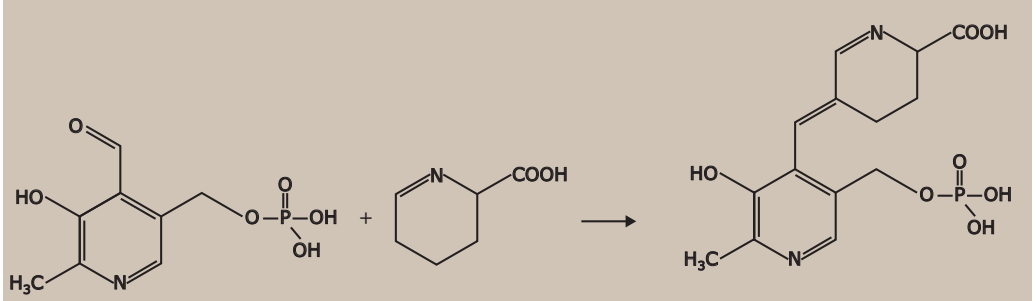
The concentrations of α-AASA and PA, in urine as well as in plasma, vary considerably in patients with PDS. A remarkable wide range of α-AASA and PA levels in patients has also been found by Mills et al in their very first report on α-AASA dehydrogenase deficiency in PDS[5]. We have no clear explanation for this wide range. Hypothetically it might reflect different levels of α-AASA dehydrogenase residual activity, dietary protein (L-lysine) intake, or the amount of pyridoxine supplemented. It is tempting to speculate that optimum treatment (i.e. pyridoxine dosage) in PDS might be achieved by focusing on the concentrations of α-AASA and PA.

## Conclusion

Metabolic investigations of urinary concentrations of α-AASA provide a reliable tool to prove PDS associated with α-AASA dehydrogenase deficiency at the metabolite level. The potentially dangerous trial of withdrawal of pyridoxine, classically used to prove PDS, can now be avoided. The novel insights into the pathophysiologic processes that underly PDS further provide us with tools to make a better estimation of the true incidence of PDS (in this study, for the Netherlands at least 1: 276 000 newborns), and will hopefully lead to an optimum treatment regime of this serious neurometabolic disorder.

**Acknowledgements**
We would like to thank all replying clinicians for their cooperation, in particular the following clinicians for kindly providing the patient data: Dr. W. Baerts, Isala Klinieken, Zwolle; Dr. F. van Berkestijn, Universitair Medisch Centrum, Utrecht; Dr. A.N. Bosschaart and Dr. R.F.H.M. Tummers, Medisch Spectrum Twente, Enschede; Dr. I. de Coo, Erasmus Medisch Centrum, Rotterdam; Dr. G.A.P.T. Hurkx, Elkerliek Ziekenhuis, Helmond; Dr. R. Kohl, Het Spittaal, Zuthpen; Dr. L.A.E.M. Laan, Leids Universitair Medisch Centrum, Leiden; Dr. A. van der Wagen, Streekziekenhuis Midden-Twente, Hengelo.

**Figure II.** Knoevenagel condensation




**PLP** **P6C** **Complex A**

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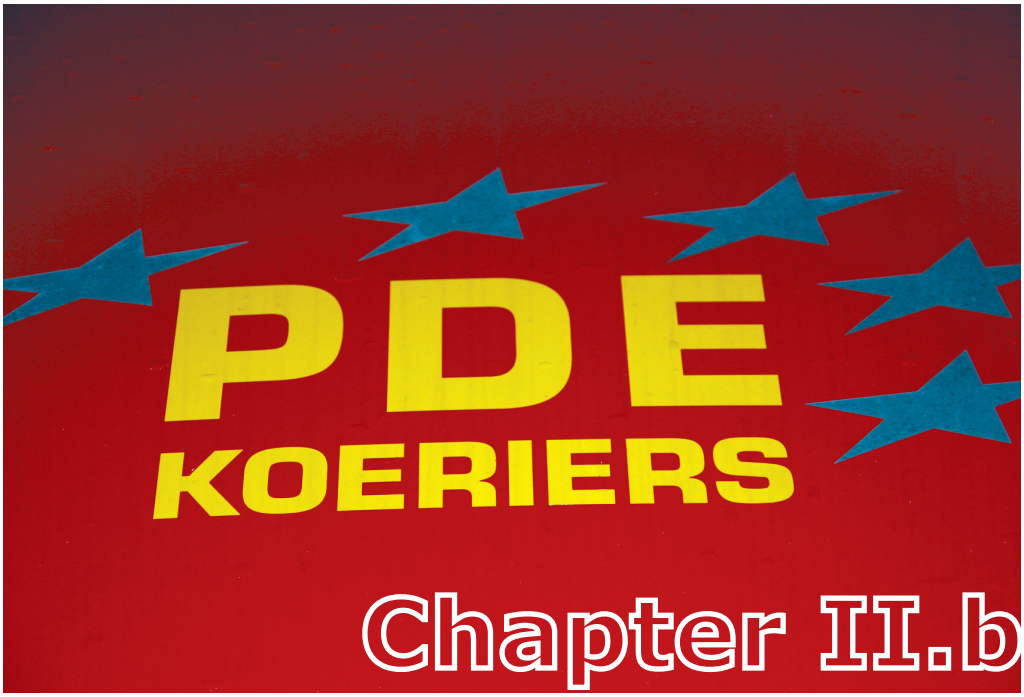



# The measurement of urinary $\Delta^1$-piperideine-6-carboxylate, the alter ego of $\alpha$-aminoadipic semialdehyde, in Antiquitin deficiency



Eduard A. Struys, Levinus A. Bok, Dina Emal, Saskia Houterman, Michèl A. Willemsen, Cornelis Jakobs

J Inherit Metab Dis 2012; 35:909-916

## Abstract

The assessment of urinary α-aminoadipic semialdehyde (α-AASA) has become the diagnostic laboratory test for pyridoxine dependent seizures (PDS). α-AASA is in spontaneous equilibrium with its cyclic form $\Delta^1$-piperideine-6-carboxylate (P6C); a molecule with a heterocyclic ring structure. Ongoing diagnostic screening and monitoring revealed that in some individuals with milder *ALDH7A1* variants, and patients co-treated with a lysine restricted diet, α-AASA was only modest increased. This prompted us to investigate the diagnostic power and added value of the assessment of urinary P6C compared to α-AASA. Urine samples were diluted to a creatinine content of 0.1 mmol/L, followed by the addition of 0.01 nmol [$^2H_9$]pipecolic acid as internal standard (IS) and 5 µL was injected onto a Waters $C_{18}$ T3 HPLC column. Chromatography was performed using water/methanol 97/3 (v/v) including 0.03 % formic acid by volume with a flow rate of 150 µL/min and detection was accomplished in the Multiple Reaction Monitoring mode: P6C: *m/z* 128.1 > 82.1; [$^2H_9$]pipecolic acid *m/z* 139.1 > 93.1. Due to the dualistic nature of α-AASA/P6C, and the lack of a proper internal standard, the method is semi quantitative. The intra CVs (n = 10) for two urine samples of proven PDS patients with only modest P6C increases were 4.7% and 8.1%, whereas their inter CVs (n = 10) were 16 and 18% respectively. In all 40 urine samples from 35 individuals with proven PDS, we detected increased levels of P6C. Therefore, we conclude that the diagnostic power of the assessment of urinary P6C and α-AASA is comparable.

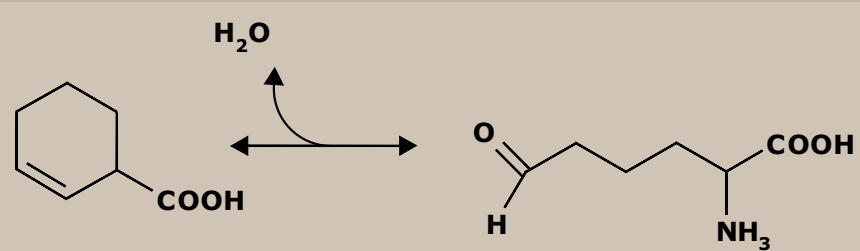
## Introduction

The finding that α-aminoadipic semialdehyde dehydrogenase (α-AASA DH/Antiquitin) deficiency is the underlying defect in the vast majority of individuals affected with pyridoxine dependent seizures (PDS), has provided the metabolic field with a specific biomarker: α-aminoadipic semialdehyde (α-AASA)[1-3]. Moreover, it has paved the way for molecular diagnosis by investigations of the corresponding gene i.e. *ALDH7A1*. Previously, the assessment of pipecolic acid in plasma and cerebrospinal fluid was the only biochemical tool for the metabolic laboratories[4,5]. A drawback of the plasma pipecolic acid assessment is that pipecolic acid can also be increased secondary to peroxisome biogenesis disorders and/or liver disease[6]. In our laboratory, we started the measurement of α-AASA in body fluids in 2005[1] (with urine being the preferred matrix), and we have detected in more than 100 individuals increases of α-AASA. In all cases, when DNA was subsequently sent to our laboratory, mutations were detected in the *ALDH7A1* gene, illustrating the specificity of the α-AASA assessment. Chemically, α-AASA is an interesting molecule, which leads a dualistic life, and this might explain why α-AASA was not picked up in the 50 years following the first description of PDS[7]. α-AASA is in spontaneous equilibrium with its cyclic form $\Delta^1$-piperideine-6-carboxylate (P6C); a molecule with a heterocyclic ring structure (Fig. I). We have prepared α-AASA out of commercially available allysine ethylene acetal[1], and the obtained product is indeed a mixture of both forms: α-AASA and P6C. This dualistic nature hampers absolute quantification, however the increases of α-AASA, also when patients were on pyridoxine supplementation, were several fold the upper limits of the age-matched control population. Ongoing diagnostic screening and monitoring in our laboratory has revealed that some individuals with milder *ALDH7A1* variants, and patients co-treated with a lysine restricted diet, displayed only modest increases.



This prompted us to investigate the diagnostic power and added value of the assessment of urinary P6C compared to α-AASA. We have developed a sensitive LC-MS/MS method which allowed us to detect P6C in non affected individuals. Urine samples were upfront diluted to a creatinine content of 0.1 mmol/L and aliquots were directly injected onto the LC-MS/MS without the need for derivatisation. Reference values were established and 40 urine samples of individuals in which α-AASA was previously found to be increased, were used for retrospective P6C quantification.

**Figure I**. Spontaneous equilibrium of P6C and α-AASA



## Material and Methods

### Urine specimens

*Reference urine samples*: The study was approved by the human medical ethics committee of Màxima Medical Centre Veldhoven. Reference urine samples (n = 91) were obtained from neonates admitted to a level IIIa neonatology intensive care unit (NICU) in Veldhoven, the Netherlands. When urine was sampled for standard neonatal care, left over material was stored at -20 °C; no additional urine samples were collected for the sole purpose of this study. From the patient's medical records, gestational age, birth weight, actual weight at urine sampling, Apgar score, protein intake, medication including anti-epileptic drugs and hypothermia treatment were documented. In all these samples α-AASA was assessed and found to be within the appropriate reference range. Reference values beyond the neonatal period were obtained by analyzing P6C levels in α-AASA negative urine samples which had been sent to our laboratory for diagnostic studies.

*Urine samples from PDS patients*: In 40 urines, stored at -20 °C, of genetically proven and urinary α-AASA positive PDS patients, we have retrospectively determined the concentration of P6C. The vast majority of these children were on pyridoxine treatment at the time of urine sampling, and in some individual cases, children were co-treated with a lysine restricted diet.

### Materials

[$^2H_9$]pipecolic acid was purchased from CDN isotopes, Pointe-Clair, Canada. All other reagents and solvents were of analytical grade. P6C was prepared out of allysine ethylene acetal (Chiralix, the Netherlands) as described before[1]. $^1$H-NMR studies of the obtained solution showed small amounts of allysine ethylene acetal, in the presence of a complicated spectrum of multiplets in the region of 1.5 to 2.5 ppm. The actual concentration of α-AASA relative to P6C could not be concluded by the obtained $^1$H-NMR data. Tandem mass spectrometer Q1 scans, in both the negative and positive electrospray ionization mode, showed signals corresponding to α-AASA and P6C, and a less abundant signal to allysine ethylene acetal (detectable in positive mode). Product ion scans (positive mode) of *m/z* 128.1 (corresponding to the M+1 signal of P6C) yielded two main fragmentation products with *m/z* 82 and *m/z* 55. Product ion scans (positive mode) of a standard solution of pipecolic acid, which can be regarded as the dihydro-analogue of P6C, showed two main fragmentation products with *m/z* 84 and *m/z* 56. All these combined data confirmed the identity of P6C, and we have optimized the LC-MS/MS using the obtained α-AASA/P6C reference solution.

### P6C sample preparation and LC-MS/MS determination

Urine samples were diluted to a creatinine content of 0.1 mmol/L with solvent A (see below), followed by the addition of 0.01 nmol [$^2H_9$]pipecolic acid serving as internal standard (IS), resulting in a final sample volume of 400 µL. Prepared samples were stable for more than 1 week at 4 °C. 5 µL was injected onto a Waters $C_{18}$ T3 HPLC column (150 x 2.1 mm, bead size 3.5 µm). Chromatography was performed using solvent A (water/methanol 97/3 (v/v) including 0.03 % formic acid by volume) with a flow rate of 150 µL/min. After elution of P6C and the IS, the column was rinsed with solvent B (water/methanol 50/50 (v/v) including

0.03 % formic acid by volume), and was subsequently allowed to regenerate with 100% solvent A. Total runtime including regeneration of the column was 7 minutes. The column was directly connected to an AB Sciex 4000 QTrap tandem mass spectrometer operating in the positive electrospray ionization mode and at optimized settings, and detection was accomplished in the Multiple Reaction Monitoring mode. The MRM transitions for P6C were: *m/z* 128.1 > 82.1 (quantifier) and *m/z* 128.1 > 55.1 (qualifier); the MRM transition for [$^2H_9$]pipecolic acid was *m/z* 139.1 > 93.1 . The P6C m/z 128.1 > 82.1 transition and the [$^2H_9$]pipecolic acid *m/z* 139.1 > 93.1 transitions correspond to the loss of the carboxyl moiety from the molecules. In addition, also the MRM transition for endogenous pipecolic acid i.e. *m/z* 130.1 > 84 was monitored. Results were calculated by directly using the obtained P6C/IS and pipecolic acid/IS peak-area ratios.

Urine spots were prepared by applying 5 µL of urine onto a Guthrie card, after which the urine was allowed to dry at room temperature and the spots were stored in the dark at room temperature. The edges of the spots were marked with a pencil, cut out by scissors, put in a small tube and dissolved in 500 µL of double distilled water. The extraction was stimulated by continuous motion of the tube for 30 minutes at room temperature, after which the tube were centrifuged at 14000 g and the supernatant was used for P6C quantification. The amount of reconstituted urine sample taken into preparations was determined by the dilution of the original urine spot relative to the volume of the extraction solvent. $\alpha$-AASA has been assessed by LC-MS/MS as described before[1]. Briefly, 1 nmol [$^{15}$N]-$\alpha$-aminoadipic acid (10 µL of aqueous 0.1 mmol/L solution) serving as internal standard, was added to 10 µL of urine. Subsequently, 100 µL of borate buffer (0.1 mmol/L, pH 10) and 100 µL of Fmoc-Cl solution (1.5 mg/ml acetone) were added, samples were mixed vigoursly, and allowed to react for 15 minutes at room temperature. 5 µL of the sample was injected onto an LC-MS/MS (as for P6C). Chromatography was performed using a Waters X-Terra C18 column (150 X 4.6 mm ID; 5 µm particle size) applying a binary linear gradient starting with 100% solvent A ($H_2O$/ACN 90/10 v/v, containing 125 mg ammonium formate) changing to 100% solvent B($H_2O$/ACN 90/10 v/v, containing 125 mg ammonium formate) in 10 minutes. Analytes were measured in negative mode using the following transitions: *m/z* -382.1 > -1861 ($\alpha$-aminoadipic acid); *m/z* -383.1 > -187.1 ([$^{15}$N]-$\alpha$-aminoadipic acid); *m/z* -366.1 > 170.1 ($\alpha$-AASA quantifier); *m/z* -366.1 > -144.1 ($\alpha$-AASA quantifier).



### Stability studies

Multiple aliquots of two urine samples of known PDS patients were stored under 3 different conditions: 1. at room temperature, 2. in the fridge at 4 °C, and 3. in the freezer at -20 °C. The samples were prepared and analyzed over a period of 40 days. Multiple Guthrie card spots from urine samples of five known PDS patients were prepared, stored in the dark at room temperature, and analyzed over a period of 30 days.

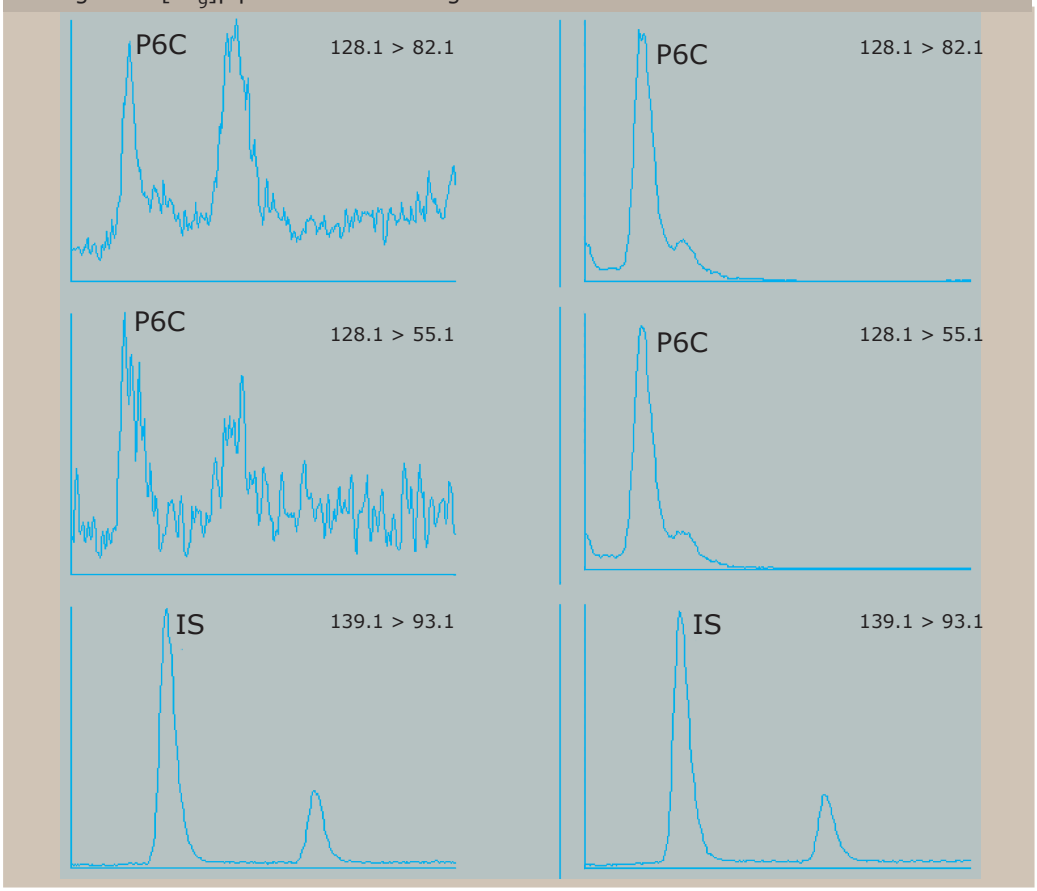
## Results

### Analytical performance of the LC-MS/MS method

The mass fragmentograms of the LC-MS/MS measurement in urine samples from a PDS positive and PDS negative individual are depicted in figure II. P6C elutes at 2.25 minutes

and [$^2H_9$]pipecolic acid at 2.50 minutes. It is important to note that due to the dualistic nature of α-AASA/P6C absolute quantification of one or the other is virtually impossible. Therefore all quantitative results presented in this study, represent estimations of the P6C concentration. For all experiments, we have chosen to use a single point calibration for the quantification of P6C, using the known concentration of the internal standard as direct reference. We made the assumption that pipecolic acid and P6C have identical mass spectrometric characteristics, although we are currently not able to substantiate this.

**Figure II.** Mass fragmentograms of the LC-MS/MS measurement of P6C; in the left panel: control urine sample (concentration 0.02 mmol/mol creatinine), and in the right panel: PDS patient urine sample (concentration 2.05 mmol/mol creatinine). IS represents the signal of [$^2H_9$]pipecolic acid serving as internal standard.



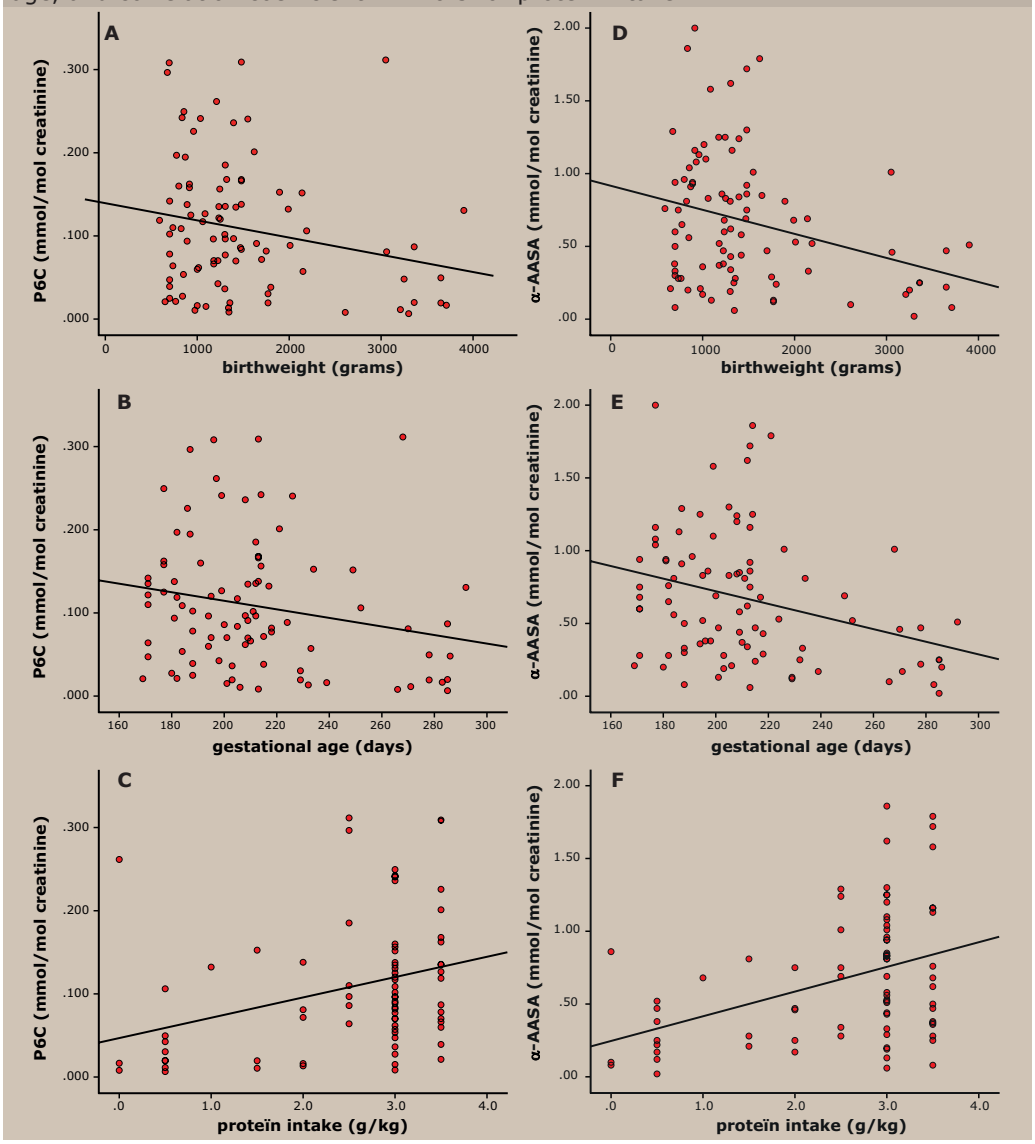



**Assay characteristics**

The intra day coefficient of variations (CVs) (n = 10) for two urine samples of two proven PDS patients with modest P6C increases were 4.7% (average ± sd: 0.38 ± 0.018 mmol/mol creatinine) and 8.1% (average ± sd: 0.68 ± 0.055 mmol/mol creatinine) ; the inter day CVs (n = 10) for these two urine samples were 16% (average ± sd: 0.35 ± 0.056 mmol/mol creatinine) and 18% (average ± sd: 0.69 ± 0.13 mmol/mol creatinine) respectively.

Linearity of the method was examined by multiple dilutions of a positive PDS urine sample with a negative urine sample with an identical creatinine concentration. The quantification of P6C was linear over the range of 1 to 30-fold dilutions, with absolute P6C concentrations ranging from 0.3 µM to 9.3 µM. The limit of detection (S/N ratio = 10) was estimated by verifying the P6C peak height relatively to the observed noise at the same chromatographic region and was found to be approximately 0.02 mmol/mol creatinine.

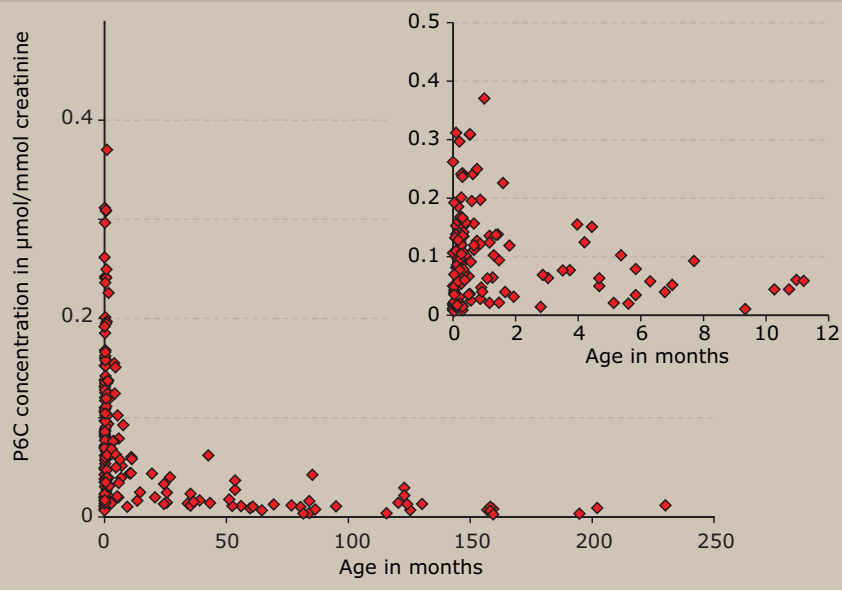
**Figures IIIA - IIIF.** P6C (A-C) and $\alpha$-AASA (D-F) levels versus birth weight (grams), gestational age (days) and protein intake (grams protein/kg body weight). For all correlations: $p < 0.05$; correlation coefficient R= -0.3 for birth weight and gestational age, and correlation coefficient R= +0.3 for protein intake.



### Stability studies

Multiple aliquots of two urines samples have been stored under three conditions. P6C was stable in both urines when samples were stored at -20 °C for 40 days. Samples stored at 4 °C showed clearly that P6C is not stable in this condition with a decrease at 40 days of 25% in urine sample A and 50% in urine sample B. P6C levels in urines stored at room temperature show a dramatic decrease of 75% for urine A to almost 100% in urine B. This decline is already notable after 5 days of storage with decreases of approximately 40%. P6C levels in urine spots on filter paper, stored in the dark at room temperature showed a decline to 50% of the original value after 10 days of storage.

**Figure IV.** P6C levels in reference population




### Urinary P6C and α-AASA reference values

Since PDS often clinically manifest in the neonatal period, 91 urine samples were obtained from neonates admitted to a level IIIa NICU in Veldhoven, the Netherlands. Samples were directly stored at -20 °C until further analysis. Additionally, 100 urine samples from individuals, in which α-AASA was normal (see reference ranges below), were used to obtain P6C reference values for all age groups. P6C and α-AASA values in newborns correlated positively with protein intake, and negatively with gestational age and body weight (Fig. IIIA - IIIF), but there was no correlation of P6C and α-AASA values with any studied neonatal IC condition, treatment, or gender. For the whole studied control population, the levels of urinary P6C showed a clear age-dependency with P6C levels up to 0.37 mmol/mol creatinine in the first months of life, decreasing to ≤ 0.05 mmol/mol creatinine for children older than 1 year (Fig. IV). We define the following urinary P6C reference ranges: ≤ 6months: P6C < 0.37 mmol/mol creatinine; > 6 months ≤ 1year: P6C ≤ 0.1 mmol/mol creatinine; > 1 year: P6C ≤ 0.05 mmol/mol creatinine. The reference ranges for urinary α-AASA are: ≤ 6months: α-AASA < 2 mmol/mol creatinine; > 6 months ≤ 1 year: α-AASA ≤ 1 mmol/mol creatinine; > 1 year α-AASA ≤ 0.5 mmol/mol creatinine. An overview

of the used methodologies for the assessment of both P6C and α-AASA are depicted in table I. As our reference population consists of an overrepresentation of premature newborns, we recommend other laboratories to in-house establish reference ranges for α-AASA and P6C.

**Table I.** Description of the used P6C and α-AASA methodologies

| Parameter | P6C method | α -AASA method |
|---|---|---|
| *Volume of urine needed* | < 50 µL | 10 µL |
| *Method* | LC-MS/MS | LC-MS/MS |
| *Internal standard* | [$^2H_9$]pipecolic acid | [$^{15}N$] α-aminoadipic acid |
| *Derivatisation* | No | Yes, FMOC-derivatisation |
| *Detection mode* | Electrospray ionization, positive mode, MRM measurement | Electrospray ionization, negative mode, MRM measurement |
| *Estimated batch (20 samples) sample preparation time* | 30 minutes | 1 hour |
| *LC-MS/MS single run time* | 7 minutes | 14 minutes |
| *Limit of quantification (S/N = 10)* | 0.02 mmol/mol cr. | 0.05 mmol/mol cr. |
| *Typical intra-assay CV's* | < 10% | < 10% |
| *Typical inter-assay CV's* | < 20% | < 20% |
| *Reference ranges ** | | |
| *0-6 months* | < 0.37 | < 2 |
| *6-12 months* | < 0.1 | < 1 |
| *> 12 months* | < 0.05 | < 0.5 |
| *Pathological values (ranges)* $* | | |
| *0-6 months* | 0.6 - 19.4 | 12 - 76 |
| *6-12 months* | 1.5 - 18.9 | 2.5 - 101 |
| *> 12 months* | 0.2 - 8.6 | 0.6 - 5.8 |

*Concentrations expressed as mmol/mol creatinine; $Pathological range of samples included in this cohort (n = 40).



## Urinary P6C levels in proven PDS patients

In a unique collection of 40 urine samples from 35 individuals with proven PDS, P6C was retrospectively assessed. In all urine samples in which previously α-AASA was found to be increased, we detected increased levels of P6C. The observed concentrations of P6C were age-dependent. We have depicted all the positive urine samples in figure V, illustrating the diagnostic performance of the measurement of urinary P6C. There is a strong relationship between the urinary levels of α-AASA and P6C in this cohort of patient samples, as is illustrated in figure VI. It is of note that in all these patient samples pipecolic acid concentrations were within the appropriate reference ranges.

**Figure V.** P6C levels in patients with proven PDS using a logarithmic Y-axis presentation

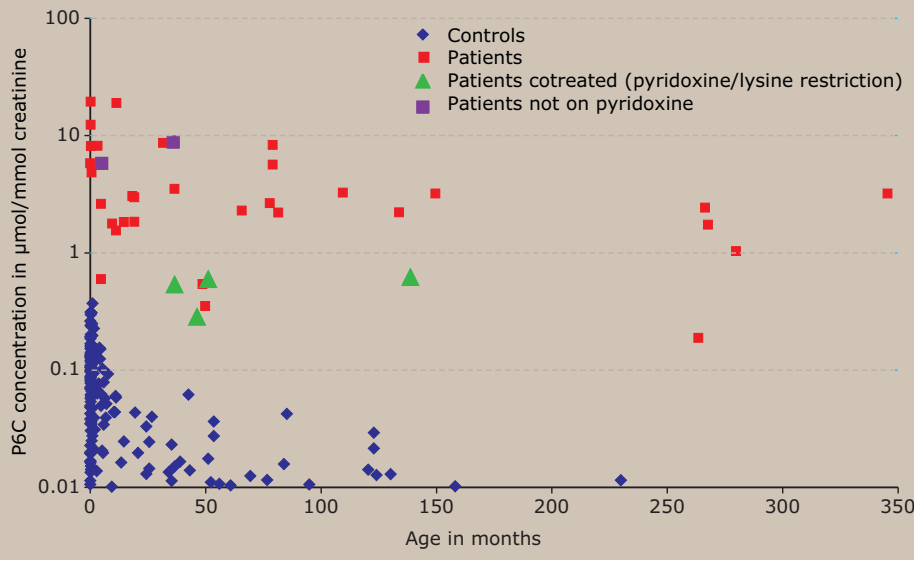
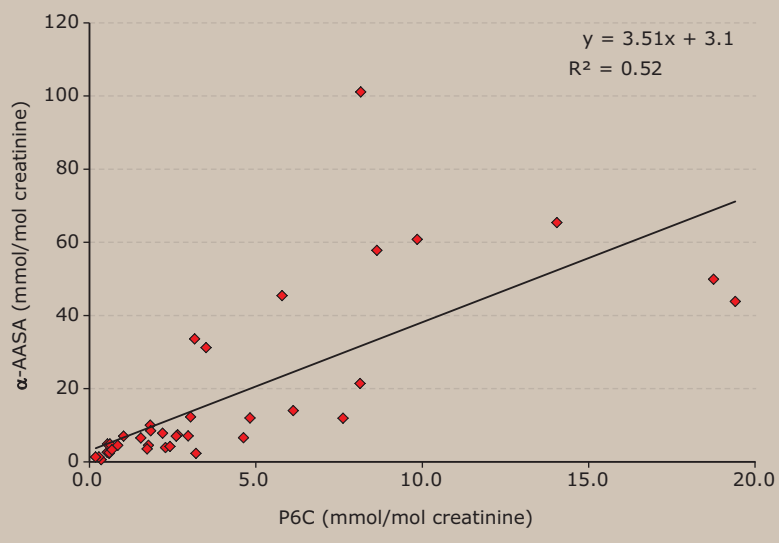



**Figure VI.** Relationship between α-AASA and P6C levels in urine samples from PDS patients; (Spearman correlation is 0.78; $p < 0.001$)






## Discussion

The differential diagnosis in neonatal and infantile seizures includes PDS. Although relatively rare, with an estimated incidence up to 1:276 000 births in the Netherlands[8], this condition is treatable and early diagnosis is of utmost importance. The current knowledge about the primary defect in PDS has provided new diagnostic biomarkers i.e. α-AASA and

P6C. Recent studies revealed that the clinical phenotype of PDS varies from classical PDS presenting as therapy resistant neonatal seizures to partly treatable seizures with the first presentation in early childhood[9]. The focus of our group, as well as others has been on the urinary levels of α-AASA, which can be detected by either LC-MS/MS or GC-MS, following a derivatisation procedure[1,10]. α-AASA is known to be in spontaneous equilibrium with its cyclic form P6C, however the mechanism of this equilibrium is poorly understood and therefore currently not inducible. Ongoing diagnostics and treatment monitoring with respect to PDS revealed that in individual cases the α-AASA increase was only modest compared to the appropriate reference range. These observations prompted us to focus on urinary P6C. P6C has previously been assessed in plasma of PDS patients and indeed, increases were detected[11]. We have chosen to investigate P6C levels in urine of patients as urinary samples can be obtained non-invasively, which is advantageous in subsequent therapy monitoring. Moreover, we have noticed that α-AASA has a strong tendency to bind with protein, and it can be expected that the same is true for P6C.

Our proposed LC-MS/MS method for the urinary P6C quantification shows sufficient sensitivity, is reproducible, and is relatively easy to perform. In an acute situation, results can be obtained within 15 minutes after urine sampling. The stability studies clearly revealed the importance of proper sample storage and shipment. The strong decline in P6C concentrations in Guthrie cards excludes this sampling procedure since there is a potential risk of false negative results.

The observed positive correlation of both P6C and α-AASA and the protein intake by neonates pleads for a lysine restricted diet therapy, thereby lowering the flux through the lysine degradation pathway, resulting in lower levels of P6C and α-AASA. Currently, we are aware that such studies are underway, however no official results have been published.



The diagnostic strength of urinary P6C and α-AASA assessments is comparable, implying that both markers can be applied in a diagnostic setting. In those cases where there is doubt about the identity of P6C or α-AASA, the alternative method can be used as a second tier test. The availability of P6C and α-AASA as biomarkers for PDS allows and encourages low-threshold screening for this treatable disorder, also in those cases where the clinical suspicion of PDS is low according to the classical criteria[12].

**Acknowledgements**
We would like to thank Erwin Jansen for his technical assistance.

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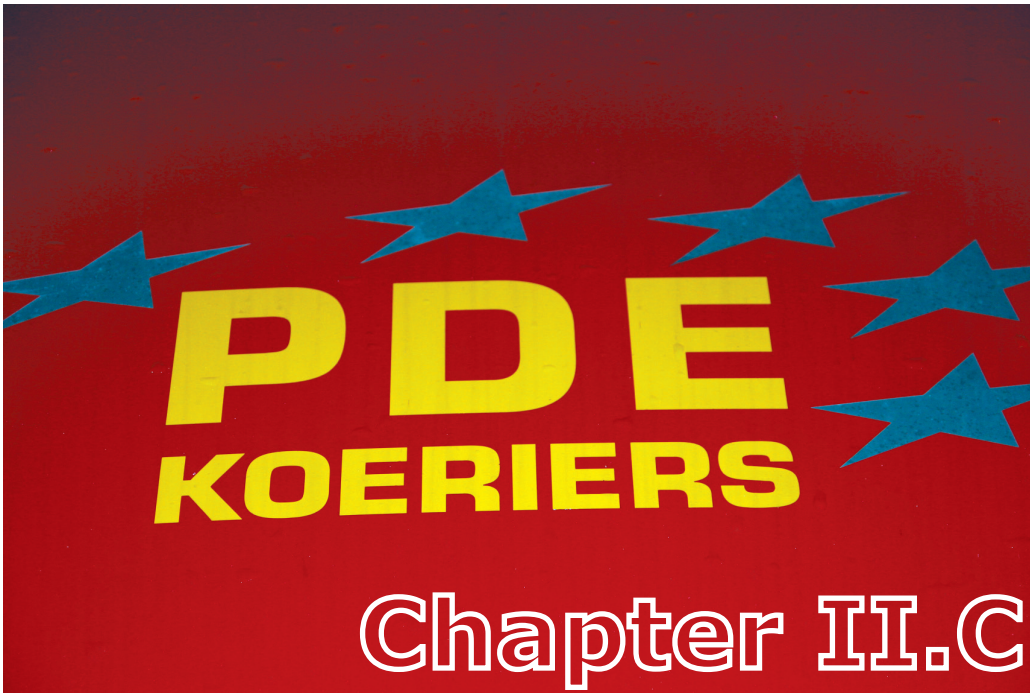



# An intriguing "silent" mutation and a founder effect in *antiquitin* (*ALDH7A1*)



GS Salomons, LA Bok, EA Struys, L Landegge Pope, PS Darmin, PB Mills, PT Clayton, MA Willemsen, C Jakobs

Ann Neurol 2007 Oct; 62(4):414-418

## Abstract

Recently, α-aminoadipic semialdehyde (α-AASA) dehydrogenase deficiency was shown to cause pyridoxine-dependent epilepsy (PDE) in a considerable number of patients. α-AASA dehydrogenase deficiency is an autosomal recessive disorder characterized by a neonatal-onset epileptic encephalopathy in which seizures are resistant to antiepileptic drugs but respond immediately to the administration of pyridoxine (OMIM266100). Increased plasma and urinary levels of α-AASA are associated with pathogenic mutations in the α-AASA dehydrogenase (*ALDH7A1/ antiquitin*) gene. Here, we report an intriguing "silent" mutation in *ALDH7A1*, a novel missense mutation and a founder mutation in a Dutch cohort (10 patients) with α-AASA dehydrogenase deficiency.

## Introduction

The first PDE patient was described more than 50 years ago[1]. However, it was not until recently that it was discovered that mutations in the α aminoadipic semialdehyde (α-AASA) dehydrogenase (*ALDH7A1*) gene cause PDE. Although patients respond immediately to the administration of pyridoxine, and often remain free of seizures during the lifelong treatment, most patients have developmental delay (OMIM266100). In all patients in whom pathogenic mutations were found, increased plasma and urinary levels of α-AASA were detected by Liquid Chromatography/mass spectrometry/mass spectrometry[2]. Currently, pathogenic mutations have been reported in 22 families, with a total of 24 mutations and 29 patients[2,3].
We have sequenced the *ALDH7A1* gene in a panel of 10 Dutch patients with biochemically proven α-AASA dehydrogenase deficiency (i.e. with increased α-AASA in body fluids).

## Subjects and Methods

### Subjects

α-AASA dehydrogenase deficiency was biochemically confirmed (i.e. increased urinary and plasma α-AASA) for 10 patients from 7 unrelated families. These biochemical data and the clinical data has been published previously[2,4,5] (Patient VI in Mills and colleagues study[2] is Patient 8 in Been and coworkers[4] and Bok and colleagues studies[5]).

### Methods

DNA was isolated from blood of patients and their parents. The 18 exons, including the adjacent splice sites of *ALDH7A1* were amplified by polymerase chain reaction (PCR) as previously described[2]. Subsequently, the purified PCR products were directly sequenced using an ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, CA, USA) and analyzed using Mutation Surveyor® (Softgenetics, PA, USA). In two cases, the *ALDH7A1* gene was analyzed at the messenger RNA level. RNA was isolated from PAXgene Blood RNA Tubes (QIAGEN Netherlands QIAGEN Benelux B.V., Venlo, the Netherlands), complementary DNA was synthesised and subsequently the full-length open reading frame was amplified using *TaKaRa LA Taq*™ polymerase (Takara Bio Europe S.A.S., Saint-Germain-en-Laye France ) and primers 1F+18R (1F; AAAGACCAGCAAGCTCTCT, 18R; CTCCAAAAACAGCTGCTGGA). The primers were designed to amplify the *ALDH7A1* mRNA only and not its pseudogene. These amplifications were directly sequenced. In addition, the amplicon harboring the homozygous mutation was cloned using the TOPO TA Cloning kit (Invitrogen, Paisly, United Kingdkom). Thirty-two individual clones were sequenced.



## Results

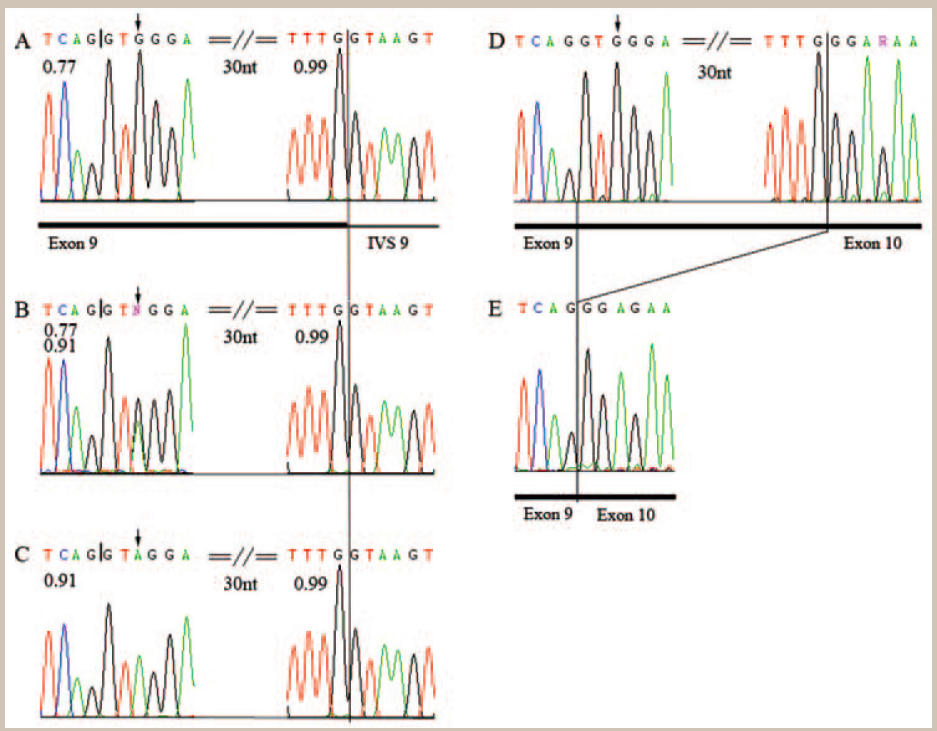
Pathogenic mutations were detected in all of the 10 patients from 7 apparently unrelated families with biochemically proven α-AASA dehydrogenase deficiency (Table I). In seven patients (four unrelated families; Patients 1,3,5,7,9,10, and 12, in the Bok and colleagues' study[5]) the c.1195G > C; p.Glu399Gln mutation in exon 14 of *ALDH7A1* was found to be homozygous; and in one patient (Patient 8), it was found to be heterozygous.

**Table I.** Overview of molecular and biochemical data of patients affected with α-AASA dehydrogenase deficiency

| Family number paper | Patient number | Subject | Mutation | Presumed effect | α-AASA urine (mmol/ mol creatinine) | α-AASA plasma (uM) | Pipecolic Acid plasma (uM) | α-AASA dehydrogenase deficiency |
|---|---|---|---|---|---|---|---|---|
| 1 | 1 | index | c. [1195G > C] + [1195G > C] | p.[Glu399Gln] + [Glu399Gln] | 16.0 | 8.0 | 6.5 | yes |
| 1 | 5 | sibling | c. [1195G > C] + [1195G > C] | p.[Glu399Gln] + [Glu399Gln] | 24.0 | 5.0 | 5.0 | yes |
| 2 | 12 | index | c. [1195G > C] + [1195G > C] | p.[Glu399Gln] + [Glu399Gln] | 39.0 | 6.1 | 7.8 | yes |
| 2 | 10 | sibling | c. [1195G > C] + [1195G > C] | p.[Glu399Gln] + [Glu399Gln] | 75.0 | 5.2 | 11.0 | yes |
| 3 | 3 | index | c. [1195G > C] + [1195G > C] | p.[Glu399Gln] + [Glu399Gln] | 29.0 | 5.7 | 4.6 | yes |
| 3 | 7 | sibling | c. [1195G > C] + [1195G > C] | p.[Glu399Gln] + [Glu399Gln] | 20.0 | 5.8 | 5.1 | yes |
| 4 | 9 | index | c. [1195G > C] + [1195G > C] | p.[Glu399Gln] + [Glu399Gln] | 9.6 | 0.8 | 22.4 | yes |
| 5 | 8# | index | c.[1195G > C] + [244C > T] | p.[Glu399Gln] + [Arg82X] | 12.0 | 2.4 | 5.3 | yes |
| 6 | 4 | index | c.[750G > A] + [750G > A] | splice errors | 4.0 | 1.1 | 7.0 | yes |
| 6 | 6 | sibling not affected | c.750G > A | splice errors | 0.2 | < 0.2 | 2.2 | no |
| 6 | | mother | c.750G > A | splice errors | - | - | - | no |
| 6 | | father | c.750G > A | splice errors | - | - | - | no |
| 7 | 2 | index | c.[1348T > A] + [c.750G > A] | p.[Cys450Ser] + [splice errors] | 4.7 | 0.9 | 5.8 | yes |
| 7 | | sibling not affected | wildtype | wildtype | - | - | - | no |
| 7 | | sibling not affected | c.1348T > A | p.Cys450Ser | - | - | - | no |
| 7 | | mother | c.1348T > A | p.Cys450Ser | - | - | - | no |
| 7 | | father | c.750G > A | splice errors | - | - | - | no |
| Control subjects | | | | | < 1 | < 0.2 | < 2.5* | |

The mutations detected in the patients and the metabolites detected in their body fluids are pipecolic acid, the previously used biomarker in plasma, and the novel biomarker aminoacid semialdehyde (AASA) both in urine and plasma. # Patient 8 is patient VI in Mills et al 2006. * Control value for patients older than 1 week. At time of sampling all patients were older than 1 week.

**Figure I.** The c.750G > A mutation results in erroneous splicing and unstable *ALDH7A1* mRNA

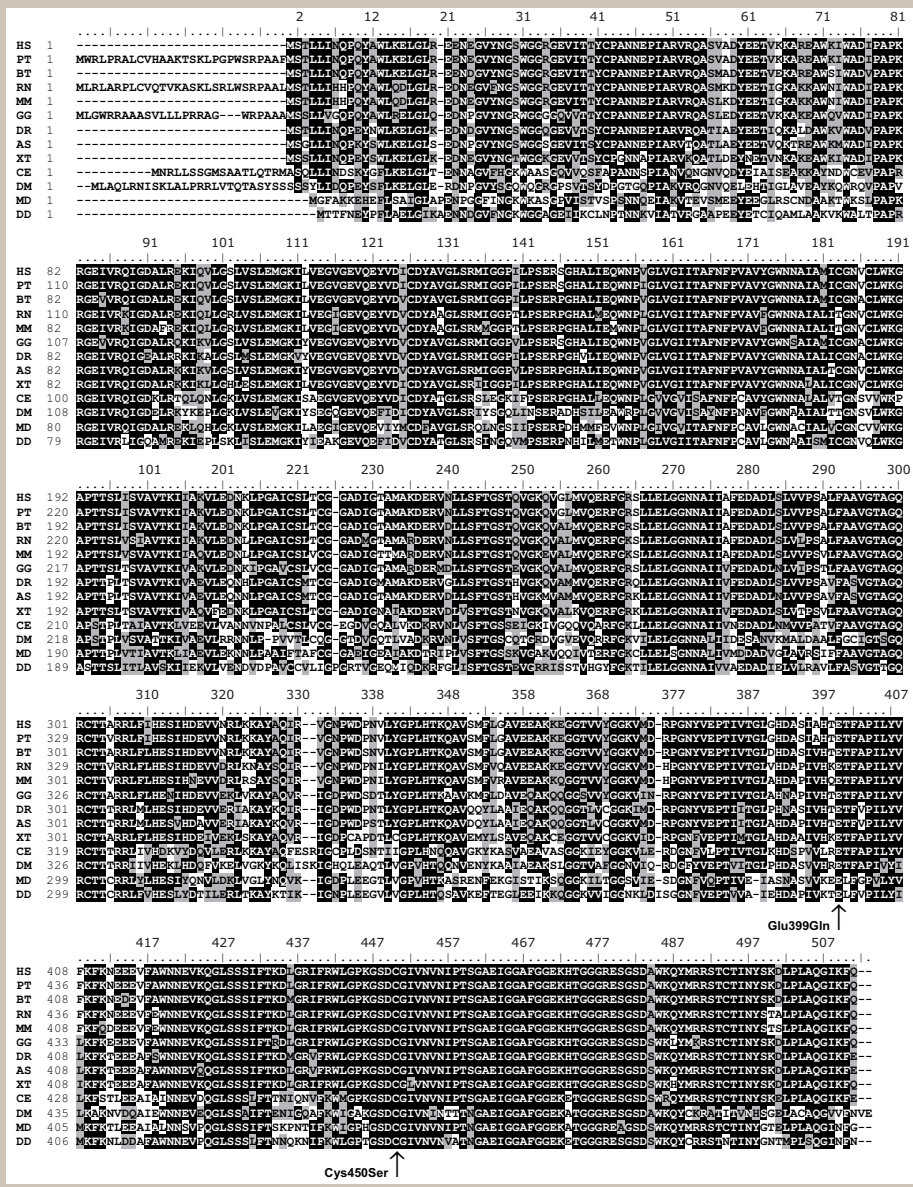



gDNA sequence analysis of 3' end of exon 9 and the donor sequence of IVS9 of the ALDH7A1 gene. A) The cryptic donor site (TCAGIGTGGA) and the authentic donor site (TTTGIGTAAGT), including the probability scores (0.77 and 0.99, respectively) in the wildtype sequence are depicted. B) In DNA of the parent both the wildtype allele as well as the allele containing the c.750G > A (N = G/C) mutation are detected (arrow), resulting in two probability scores for the cryptic upstream donor site (0.77 and 0.91). C) In DNA of the patient only the homozygous transition (c.750G > A) is present, resulting in the increase of the probability score to 0.91 for the cryptic donor site (TCAGIGTGGG; 0.91), but without a predicted effect on the authentic donor site 40 nt upstream. Correct splicing (D) and erroneous splicing (E) of *ALDH7A1* mRNA isolated from blood of control and patient, respectively. In the control, only mRNA spliced at the authentic donor site is detected. No difference between this control RT-PCR and that of the parents was seen, indicating that mRNA spliced at the upstream donor site probably results in instable mRNA. The arrow indicates the position of the nucleotide involved in the mutation (see A-D).



A novel homozygous "silent" variant/mutation (c.750G > A) was detected in one of the patients (Patient 2). Her parents were carriers of this "silent" variant/mutation, confirming homozygosity in the affected child. The biochemically unaffected sibling (i.e. normal $\alpha$-AASA levels) was heterozygous for the mutation like her parents. The mutation was not detected in 210 control chromosomes. The splice prediction tool of Berkely Drosophila Genome project (http://www.fruitfly.org/seq_tools/splice.html) suggested that in the wild-type sequence, 40 nucleotides upstream of the authentic donor site of IVS9, a cryptic donor site is located. The authentic donor site has an extremely high probability score of 0.99 (score varies from 0.1 - 1), but also the cryptic site has a reasonable score of 0.77 (Fig. I), which suggests that both sites may be used. The silent mutation is located within the cryptic site, resulting in an increase of its predicted score to 0.91. This does not affect the authentic

**Figure II.** Box alignment of ALDH7 proteins




Alignment was determined by the ClustalW BioEdit, CA USA program using the ALDH7A1 proteins that were identified to be most related to the homo sapiens ALDH7A1 protein by the blastp search. The boxshade program was used to visualize identical amino acids (highlighted in black) and functionally conserved amino acids (in grey). The arrows point at the two amino acids involved in the missense mutations detected in the present study. Functionally conserved amino acids are classified as follows: V, I, L, and M; D, E, Q, and N; F,Y, and W; G, S, T, P, and A; and K, R, and H. HS Homo sapiens GI 4557343, PT Pan troglodytes GI 114601421, BT Bos Taurus GI 114051810, RN Rattus norvegicus GI 62664437, MM Mus musculus GI 74219152, GG Gallus gallus GI 118104602, DR Danio rerio GI 47086597, AS Acanthopagrus schlegeli GI 61742178, XT Xenopus tropicalis GI 62858515, CE Caenorhabditis elegans GI 115534176, DM Drosophila melanogaster GI 24666674, MD Malus x domestica GI 25090068, DD Dictyostelium discoideum GI 66818493.

donor site. Similar results were obtained when other splice prediction tools were used (e.g. www.genet.sickkids.on.ca/~ali/splicesitefinder.html). RNA isolated from the blood of patient, parents and controls followed by RT-PCR and sequencing of the cDNA demonstrated that only the authentic site appears to be used in the control. However, analysis of the RNA from our patient, who is homozygous for the c.750G > A variant, demonstrated that the cryptic site is preferentially used over this authentic site. In contrast, RNA isolated from blood of the parents, who are heterozygous for the mutation, showed only the presence of properly spliced mRNA. This suggests that the eroneous splicing results in mRNA that could be subjected to non-sensense-mediated decay[6], resulting in the abundance of authentic spliced transcripts over the aberrant spliced transcripts. The low abundant aberrant mRNA is the only form present in the index, allowing this to be amplified by PCR in contrast with the heterozygotes where the high abundane of authentic spliced transcripts probably interferes. The cloning of the amplicons showed that 29 of 32 clones that could be analyzed had a deletion of the last 40 nucleotides of exon 9 (r.748_787del), arising from the cryptic donor site (c.749) decribed earlier. This predicts a framshift leading to a new stop codon 23 amino acids downstream of the valine (p.Val250GlyfsX23). The three remaning clones harbored the authentic spliced sequence, indicating that normal splicing also occurs at a lower rate.

In DNA of Patient 4, the heterozygous splice mutation described earlier (c.750G > A) and a novel heterozygous missense variant were detected. Sequence analysis of the complete open reading frame at the complementary DNA level showed only the presence of a properly spliced allele containing the novel missense variant (c.1348T > A; p.Cys450Ser). The cysteine residue as well as the protein region is highly conserved in evolution (Fig. II). The missense variant was not detected in 210 control chromosomes. Compound heterozygosity for both alleles was only detected in the affected sibling and not in the two unaffected siblings and/or the parents.



## Discussion

The c.1195G > C; p.Glu399Gln mutation was detected in the majority of the Dutch $\alpha$-AASA dehydrogenase (antiquitin) alleles. It has been reported that this mutation occurs in 13 out of 48 alleles (24 index patients) showing that this mutation has a high frequency[2,3] (Table I). The fact that this mutation was detected in 9 out of 14 alleles from apparently unrelated Dutch index patients (including a previously described Dutch allele[2]) is a strong argument for a founder effect.

In two Dutch families, a novel "silent" mutation (c.750G > A) was found, a type of DNA variation that is often considered to be nonpathogenic because the coding sequence and/or protein function is thought to be altered. Interestingly, this variant proved to be the pathogenic mutation, because it results in erroneous splicing (Fig. I). This is in agreement with the absence of the mutation in control chromosomes, the data obtained with the free web-based splice prediction tools, and the segregation of the homozygous mutation with the clinical phenotype within the family. Although highly speculative, it is of note, that in the patient who is homozygous for this "silent" mutation, both urinary and plasma

AASA levels (4.0 mmol/mol creatinine and 1.1 umol/L, respectively), although increased compared to controls, appear to be moderately increased compared to those found in other Dutch patients (n = 10: range 9.6 - 75 mmol/mol creatinine, and 0.8 - 8.0 umol/L, respectively). This may suggest the presence of very low levels of properly spliced mRNA, which would be expected based on the presence of the unaffected authentic splice site and was confirmed by the fact that 3 of 32 (approximatly 9%) clones contained the authentic spliced mRNA.

Furthermore, this mutation has also been detected in another patient. This "silent" mutation (c.750G > A, heterozygous) described above, was found in conjunction with a novel heterozygous missense mutation that results in the replacement of a cysteine by a serine (p.Cys450Ser). The latter is considered pathogenic based on the following 4 arguments: (1) the cysteine residue and the protein region are highly conserved throughout evolution (Fig. II); (2) the missense mutation was not detected in 210 control chromosomes; (3) compound heterozygosity for both alleles was detected only in the affected sibling and not in the 2 unaffected siblings and both parents; and (4) no additional mutations or splice aberrations were detected in the mRNA, making it unlikely that another mutation had been missed. It is notable that also in this patient, the increase of $\alpha$-AASA levels (urine 4.7 mmol/mol creatinine, plasma 0.9 umol/L) is modest compared to other $\alpha$-AASA dehydrogenase deficient patients (n = 10: range; urine 9.6 - 75 mmol/mol creatinine, plasma: 0.8 - 8.0 umol/L). However, the limited number of patients tested does not allow any definitive conclusion on these metabolite levels, and further enzyme studies are warranted.

The study further emphasizes that elevated urinary $\alpha$-AASA is associated with pathogenic mutations in *ALDH7A1*. This illustrates that increased $\alpha$-AASA levels should be used as a non-invasive pathognomonic marker in diagnostic laboratories. It may be desirable, at least in the Netherlands, but likely in a broader area, first to analyze the DNAs for the presence of mutations in exons 14 (p.Gln399Glu), 9 (c.750G > A) and 4 (p.Arg82X) of *ALDH7A1*, before sequencing the complete open reading frame (i.e. an additional 15 exons).



Furthermore, we detected an intriguing "silent" mutation that led to the introduction of a cryptic splice site that predicts to encode a truncated protein. Notably, a silent variant may also have an effect on *cis*-elements, resulting in erroneous splicing[6], or it may even lead to different kinetics of mRNA (protein) translation[7]. This study illustrates the importance of mRNA studies when a seemingly nondisease-causing variant is detected or in the case where there is a strong suspicion of $\alpha$-AASA dehydrogenase deficiency (i.e. increased urinary levels of $\alpha$-AASA) without the identification of one or both mutated *ALDH7A1* alleles. The fact that *ALDH7A1* is expressed in blood allows the inclusion of mRNA studies in such occasions.

**Acknowledgements**
We acknowledge Drs C. Martinez Munoz, E.H. Rosenberg and A. Errami for critically reading the manuscript. We also greatly acknowledge M. Fernandez Ojeda fot the excellent cloning experiments.

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# Clinical Studies in PDE


# The EEG response to pyridoxine-IV neither identifies nor excludes pyridoxine-dependent epilepsy



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Epilepsia 2010 Dec; 51(12):2406-2411

## Abstract

**Purpose**: Pyridoxine Dependent Epilepsy (PDE) is characterized by therapy resistant seizures (TRS) responding to intravenous (IV) pyridoxine. PDE can be identified by increased urinary $\alpha$-AASA concentrations and mutations in the *ALDH7A1 (antiquitin)* gene. Prompt recognition of PDE is important for treatment and prognosis of seizures. We aimed to determine whether immediate electroencephalography (EEG) alterations by pyridoxine-IV can identify PDE in neonates with TRS.

**Methods**: In 10 neonates with TRS, we compared online EEG alterations by pyridoxine-IV between PDE (n = 6) and non-PDE (n = 4). EEG segments were visually and digitally analyzed for average background amplitude and total power and relative power (background activity magnitude per frequency band and contribution of the frequency band to the spectrum).

**Results**: In 3 of 10 neonates with TRS (2 of 6 PDE and 1 of 4 non-PDE neonates), pyridoxine-IV caused flattening of the EEG amplitude and attenuation of epileptic activity. Quantitative EEG alterations by pyridoxine-IV consisted of (1) decreased central amplitude, $p < 0.05$ [PDE: median -30% (range -78 to -3%); non-PDE: -20% (range -45 to -12%)]; (2) unaltered relative power; (3) decreased total power, $p < 0.05$ [PDE: -31% (-77 to -1%); -27% (-73 to -13%); -35 % (-56 to -8%) and non-PDE: -16% (-43 to -5%); -28% (-29 to -17%); -26% (-54 to -8%), in delta-, theta- and beta-frequency bands, respectively]; and (4) similar EEG responses in PDE and non-PDE.

**Discussion**: In neonates with TRS, pyridoxine-IV induces non-specific EEG responses that neither identify nor exclude PDE. These data suggest that neonates with TRS should receive pyridoxine until PDE is fully excluded by metabolic and/or DNA analysis.


**Keywords**: Neonatal seizures, Electroencephalography, Antiquitin, *ALDH7A1* gene mutations, Pyridoxine.

## Introduction

For accurate treatment of neonatal seizures, it is important to first ascertain underlying etiology[1]. Under some conditions (such as pyridoxine dependent epilepsy (PDE), pyridoxine 5'-phosphate oxidase deficiency (PNPO) and folinic acid responsive seizures), therapy resistant seizures (TRS) may respond to administration of pyridoxine (vitamin B6) and/or pyridoxal phosphate[2-5]. The incidence of PDE is relatively low (1:300 000 newborns in the Netherlands[6]). Classical presentations of PDE concern early, intractable neonatal seizures that cease after pyridoxine administration[7,8]. PDE is caused by an inherited metabolic disorder of lysine degradation, resulting in increased urinary alpha-aminoadipic semialdehyde ($\alpha$-AASA) excretion. In addition to increased urinary $\alpha$-AASA concentrations, mutations in the *ALDH7A1 (antiquitin)* gene can also identify PDE[9-14]. Although prognosis may vary even after early treatment[15], it is conceptualized that fast and accurate PDE treatment could be beneficial[16-19].

To avoid delay by biochemical and genetic testing, diagnostic trajectories often include an empirical pyridoxine trial. Such pyridoxine trials generally involve assessment of the clinical response to pyridoxine administration and subsequent withdrawal[20-22]. In TRS patients, there are two common routes for pyridoxine administration: (1) The intravenous (IV) route, which is generally associated with fast clinical and EEG responses, and (2) the oral route, which is generally associated with slower clinical and EEG responses[17,20]. In perspective of the faster response, the IV-route is often applied in neonates with a high seizure frequency.

Prior to pyridoxine-IV, neonatal EEG signals have been characterized by a discontinuous background activity[23-25], with bilateral high-voltage delta waves and with central/temporal (poly)spikes[24]. After pyridoxine-IV, epileptic activity may disappear instantaneously[26,27]. This effect can be accompanied by other EEG characteristics, such as flattening of the EEG trace[23,24,28]. However, pyridoxine-IV EEG responses can vary among PDE patients[25], and the specificity of EEG signals can be affected by many different anti-epileptic drug (AED) treatments that children with PDE often receive prior to pyridoxine-IV[23,29]. In addition, one should also take into account that EEG signals in PDE neonates would be expected to differ from EEG signals in older children with PDE[8]. Finally, it is indicated that pyridoxine-IV may also induce a favourable anti-epileptic effect in absence of PDE[30]. Accordingly, we have shown that pyridoxine-IV can nonspecifically reduce the EEG amplitude in non-PDE neonates with TRS[31], we therefore reasoned that non-specific EEG effects could hamper the clinical interpretation of the pyridoxine-IV trial.

Given the preceding, we aimed to investigate whether direct EEG responses to pyridoxine-IV allow prompt identification of neonatal PDE. For this purpose, we compared quantitative (digital) and qualitative (visual) EEG responses to pyridoxine-IV between neonates characterized by PDE (by *ALDH7A1 gene mutation*) and neonates characterized by non-PDE. To the best of our knowledge, such quantitative EEG responses to pyridoxine-IV have not been systemically investigated before in a homogeneous neonatal PDE cohort.

## Patients

In TRS (PDE and non-PDE) neonates, we retrospectively collected and compared EEG recordings during first pyridoxine-IV exposure. The medical ethical committee of the University Hospital Groningen approved the present study. TRS were defined as seizures that do not respond to adequate administration of at least two first-line AEDs. Prescibed AED prior to the pyridoxine-IV administration involved therapeutic dosages of benzodiazepines, phenytoin, lidocaine and/or phenobarbital (Table I). PDE was defined according to the clinical criteria of Baxter[22] and confirmed by *ALDH7A1* gene mutation analysis[9,13]. All PDE data were derived from the Dutch study cohort (until January 2010 consisting of a total of 15 PDE children; see for further description Bok et al.[9]). For analysis of PDE data, we selected all six neonatal (< 3 months of age) EEG recordings that were obtained during first pyridoxine-IV exposure. All six PDE neonates were born, treated, and recorded between 1999 and 2008, at five different academic hospitals in the Netherlands (Academic Medical Center Amsterdam; Erasmus Medical Center Rotterdam; University Medical Center Groningen; University Medical Center Nijmegen; University Medical Center Utrecht). After parental informed consent (2008), we retrospectively performed EEG analysis in PDE neonates at the University Medical Center Groningen (between 2008 and 2009). Except for PDE neonate 6, all other (n = 5) included PDE neonates were homozygous for the common Dutch- c.1195G > C mutation in *ALDH7A1*. PDE neonate 6 appeared compound heterozygous with a c.1195G > C mutation and a c.244C > T mutation[13]. In all PDE neonates, urine $\alpha$-AASA levels were increased (≥ 9 mmol/mol creatinine; controls < 1 mmol/mol creatinine), whereas in all non-PDE neonates urine $\alpha$-AASA levels were normal.

All non-PDE neonates were born, treated, and recorded between 2001 and 2004 at the University Medical Center Groningen [see for patient characteristics[31]]. After parental informed consent (obtained in 2005), we retrospectively performed non-PDE EEG analysis at the University Medical Center Groningen[31]. One of the analyzed pyridoxine responsive TRS neonates (former case 3) was definitely diagnosed with PDE and was, therefore assigned to the present PDE group. We consecutively obtained pyridoxine-IV EEG data for four remaining non-PDE neonates [< 3 months of age; i.e., former cases 1, 2 5, and 6[31]]. The above described patient selection criteria resulted in relatively homogeneous PDE and non-PDE characteristics, both regarding seizure onset (0 - 2 days after birth) and regarding the timing of EEG recordings (2 - 15 days after birth). Patient data are shown in table I.



## Methods

In all 10 included neonates with TRS, we visually analyzed EEG recordings during the first pyridoxine-IV administration. All TRS neonates received 100 mg pyridoxine-IV, except for one PDE neonate and one non-PDE neonate who received 50 and 300 mg, respectively. Both 5 - 15 minutes before and after pyridoxine-IV, we obtained two one-minute EEG segments involving identical vigilance states. Segments were excluded for artifacts. All EEGs of TRS neonates were subsequently assigned and compared between PDE and non-PDE subgroups.

**Table I.** Clinical data of neonates with therapy-resistant seizures (TRS)

| | Infant | Signal | AED at pyridoxine-IV | Seizure classification | Seizure type | Seizure onset | Age Pyrid-IV | Urine conc. AASA |
|---|---|---|---|---|---|---|---|---|
| PDE | 1 | D | B, PB | G | Myoglonic | 0 d | 3 d | 9.6 |
| | 2 | D | B, LD | MF | Miscelaneous | 0 d | 3 d | 71 |
| | 3 | D | B, PB, P | G | Tonic | 0 d | 15 d | 32 |
| | 4 | D | PB, P | G | Myoclonic | 0 d | 2 d | 24 |
| | 5 | aEEG | B, PB, LD | MF | Miscelaneous | 2 d | 7 d | 32 |
| | 6 | P | B, PB, P | MF | Toni Clonic | 0 d | 3 d | 12 |
| Non-PDE | 1 | D | B, PB | C, G | Toni clonic | 0 d | 12 d | < 1 |
| | 2 | D | B, PB, LD | S, G | Subtle | 0 d | 2 d | < 1 |
| | 3 | D | B, PB | S, G | Toni Clonic | 1 d | 7 d | < 1 |
| | 4 | D | B, LD, PB | S, G | Tonic clonic | 1 d | 13 d | < 1 |

PDE, pyridoxine dependent epilepsy; AED, antiepileptic drug; IV, intravenous; conc., concentration; AASA, alpha-aminoadipic semialdehyde in mmol/mol creatinine (normal value < 1); D, digital; aEEG, amplitude integrated EEG; B, benzodiazepines; P, phenytoin; LD, lidocaine; PB, phenobarbital; C, cryptogenic; S, symptomatic; G, generalized; MF, multifocal; d, postnatal day(s).

### Visual EEG analysis

We visually compared the EEG response during pyridoxine-IV between neonatal PDE and non-PDE (six and four neonates, respectively). EEG recordings of PDE neonates consisted of four digital 21-channel registrations, one digital amplitude integrated EEG (aEEG) registration and one paper 21-channel registration. EEG recordings in non-PDE neonates consisted of four digital 21-channel EEG registrations.

### Quantitative analysis of digital EEG recording

We assessed the same EEG segments for both digital and visual analysis. In eight TRS neonates, we digitally analyzed eight EEG recordings for amplitude, total and relative power (in delta, theta and beta frequency-bands). Quantitative EEG data of TRS neonates were subsequently subdivided and compared between PDE (n = 4) and non-PDE (n = 4) subgroups.

Total power reflects the magnitude of background activity for an individual frequency band. Relative power indicates the relative contribution of a frequency-band to the entire spectrum[32]. Digital EEGs were analyzed by Brainlab (version 4.00-0.00) and Brain Vision Analyzer (version 1.030002). Bipolar recordings were evaluated at frontal (F3-F4), centro-temporal (T3-C3;C4-T4), central (C3-C4) and occipital (O1-O2) electrodes. The mean amplitude was calculated for each segment of 60 seconds (sampled at 256 Hz), after rectification. For power calculations, we applied a Fast Fourier Transform algorithm, as implemented in Brain Vision Analyzer, employing a 10% Hanning window and using segments of 60 s, resulting in a spectral resolution of 0.016 Hz. Before and after

pyridoxine-IV, we compared relative power in each frequency-band. Relative power calculation was performed according to the formula ($P_{relative}$ [f1, f2] = P[f1, f2] / P[0.50] x 100%)[31], where P[f1, f2] is the total power in the frequency-band between f1 and f2 Hz.[32].

### Statistical analysis

Before and after pyridoxine-IV, we applied Wilcoxon signed rank test to assess EEG alterations (for amplitude, total- and relative- power). We applied Mann-Whitney U-test to compare quantitative EEG alterations (regarding amplitude, total- and relative- power) between PDE and non-PDE subgroups. We stratified results for vigilance state (i.e. sleeping or waking) by Mann-Whitney U-test. The level of significance was set by $\alpha = 0.05$.

## Results

### Visual EEG assessment

We observed a flattening of the EEG response (i.e. iso-electrical trace) and attenuation of epileptic activity in two of six neonatal PDE trials and in one of four non-PDE trials. Both 15 minutes before and after pyridoxine-IV, visual assessment did not reveal diagnostic EEG features that can identify PDE or non-PDE in a sensitive or specific was. The recording of the aEEG in the PDE neonate showed a continuous normal voltage pattern with normal sleep cycles. Until 48 hour after pyridoxine administration, we did not observe a clear aEEG response.

### Quantitative analysis of digital EEG recordings

*Amplitude:*

Digital EEG responses to pyridoxine-IV administration were assessed at central, centro-temporal, frontal and occipital electrodes. In eight TRS neonates, EEG amplitudes declined at central, centrotemporal, and frontal electrodes ($p < 0.05$) [eight trials; C3-C4 median -20% (-82 to -3 %; Fig. Ia); C3-T3: -21% (-78 to -2%); C4-T4: median -17% (-76 to -5%); F3-F4: median -6% (-64 to -2%)]. After subdivision according to PDE and non-PDE, EEG amplitude alterations similarly declined in both PDE (central decline in PDE: median -30% (-78 to -3%; Fig. Ib); and in non-PDE: median: -20% (-45 to -12%; Fig. Ic). At occipital electrodes, EEG amplitude alterations did not significantly change. Regarding EEG amplitude alterations, stratification according to vigilance levels did not show different responses between PDE and non-PDE.



*Total power:*

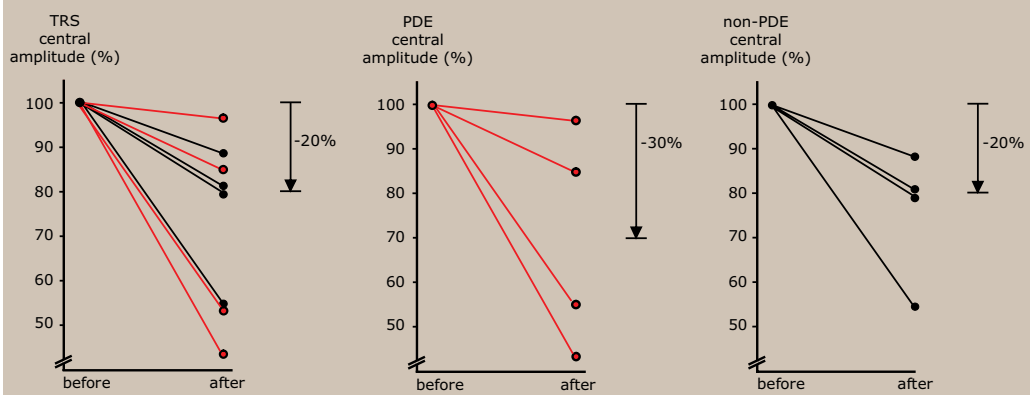
In all neonates with TRS, we separately assessed the effect by pyridoxine-IV on total power in delta, theta and beta frequency bands (Fig. II). This revealed a decline in total power for all frequency bands at central, centro-temporal and frontal electrodes (median declines -21%, -17%, -17% respectively; $p < 0.05$). Subdivision according to PDE and non-PDE did not show different responses in total power between both groups [total power decline at C3C4 medians (ranges) in: (1) delta-band, PDE: -31% (-77 to -1%) and non-PDE: -16%, (-43 to -5%); (2) theta-band, PDE: -27% (-73 to -13%) and non-PDE: -28% (-29 to -17%); and (3) beta-band, PDE: -35% (-56 to -8%) and non-PDE: -26% (-54 to -8%)]; Fig. IIa-c. At occipital electrodes, total power did not decline in neither of the groups. Stratification

according to vigilance levels did not reveal different outcomes in total power between PDE and non-PDE.

*Relative power:*
In neonates with TRS (both PDE and non-PDE), pyridoxine-IV did not affect relative power (percentages before versus after pyridoxine for delta, theta and beta-band: in PDE: 50% versus 48%; 33% versus 33% and 16% versus 17% and in non-PDE: 57% versus 59%; 26% versus 26% and 15% versus 14%, respectively). Relative power did not significantly differ between neonates with PDE and non-PDE. Regarding relative power alterations, stratification according to vigilance levels and pyridoxine dosages did not show different responses between PDE and non-PDE.

**Figure I**. Decline in EEG amplitude by pyridoxine-IV in TRS infants ($p < 0.05$). Central amplitude responses to pyridoxine-IV are separately indicated for infants with TRS (Fig. Ia); PDE (Fig. Ib) and non-PDE (Fig. Ic). At the vertical axis, central EEG amplitude alterations are expressed as percentile changes against individual reference values (of 100%) before pyridoxine IV. At the horizontal axis, administration of pyridoxine IV is indicated. The median decline is indicated by an arrow (solid line), at the right side of figures Ia-c. The inter individual variability of EEG responses to pyridoxine-IV prohibits distinction between PDE (red) and non-PDE (black).

**Central amplitude decline ($p < 0.05$) upon pyridoxine-IV in TRS (subdivided for PDE and non-PDE)**



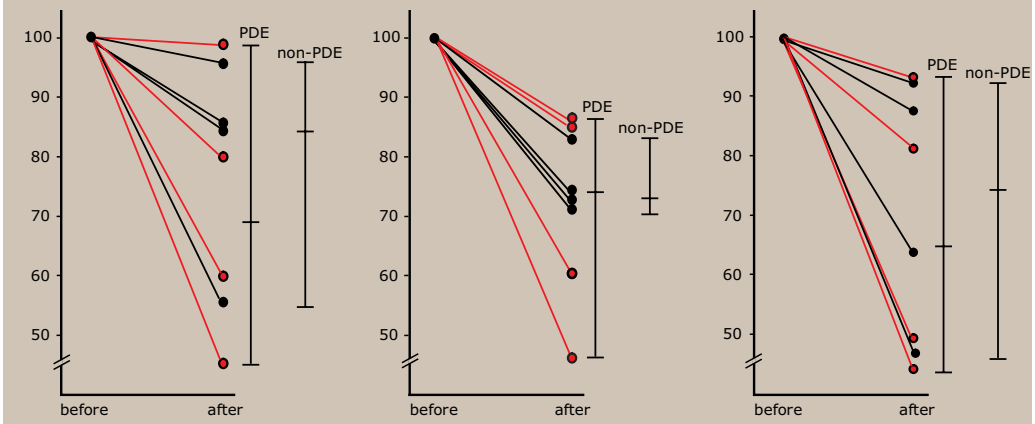

**Ia.** TRS infants: inter-individual % amplitude alteration (median decline 20%; $p < 0.05$). Open circles indicate PDE neonates, closed circles indicate non-PDE neonates.

**Ib.** PDE: inter-individual % amplitude alteration (median decline 30%).

**Ic.** non-PDE: inter-individual % amplitude alteration (median decline 20%).

**Figure II**. Decline in total power by pyridoxine-IV in TRS infants ($p < 0.05$). At central electrodes (C3C4), individual alterations are expressed for delta, theta and beta frequency bands (Fig. IIa, b and c). Medians and ranges are separately indicated for PDE (red vertical line) and non-PDE (black vertical line), at the right side of figures IIa-c. In all frequency bands, total power responses to pyridoxine-IV overlapped and did not distinguish between PDE and non-PDE.

**Total power decline ($p < 0.05$) upon pyridoxine-IV in TRS (medians and ranges for PDE and non-PDE)**




**IIa.** delta band: inter individual % of total power decline in TRS neonates (median decline 18%; $p < 0.05$). Vertical lines indicate ranges & medians of PDE and non-PDE. Open circles indicate PDE neonates, closed circles indicate non-PDE neonates.

**IIb.** theta band: inter-individual % of total power decline in TRS neonates (median decline 27%; $p < 0.05$). Vertical lines indicate ranges & medians of PDE & non-PDE. Open circles PDE neonates, closed circles indicate non-PDE neonates.

**IIc.** beta band: inter-individual % of total power decline in TRS neonates (median decline 28%; $p < 0.05$). Vertical lines indicate ranges and medians of PDE & non-PDE. Open circles indicate PDE neonates, closed circles indicate non-PDE neonates.

## Discussion



In neonates with TRS, we aimed to determine whether the EEG response to pyridoxine-IV can identify PDE by *ALDH7A1* gene mutations. To the best of our knowledge, quantitative pyridoxine-IV EEG responses have not been systematically investigated before in a homogeneous neonatal PDE cohort. Present results indicate that pyridoxine-IV induces EEG alterations that are neither highly sensitive nor specific for PDE by *ALDH7A1*gene mutations.

The *ALDH7A1* gene encodes for the enzyme α-aminoadipic semialdehyde (α-AASA) dehydrogenase, which is involved in lysine degradation. α-AASA dehydrogenase deficiency causes increased levels of α-AASA in body fluids[11]. α-AASA is in spontaneous equilibrium with its cyclic form i.e. L-delta(1)-piperideine-6-carboxylate, and the latter is able to "trap" pyridoxal-5-phosphate, resulting in pyridoxal-5-phosphate deficiency[11]. Pyridoxal-5-phosphate is a cofactor involved in many metabolic pathways of the brain[27]. Its function as co-factor for the enzymatic conversion of (excitatory) glutamate into (inhibitory) GABA[16,18,33] could explain why pyridoxine supplementation may attenuate seizures in general (i.e. in non-PDE patients). However, it is indicated that CSF glutamate and/or GABA concentrations are not unanimously associated with the pyridoxine response[27]. In

accordance with this observation, present digital EEG data did not reflect enhancement of specific GABA-ergic characteristics after pyridoxine-IV administration (such as for instance increased beta activity[34]). Hopefully, future studies may further clarify the underlying mechanism for seizure reduction by pyridoxine.

In neonatal PDE, direct EEG alterations by pyridoxine-IV are characterized by attenuation of seizure activity and flattening of the trace[23,24]. However, in the present study, four of six PDE neonates lacked such a direct EEG pyridoxine-IV response. Interestingly, one of the non-PDE neonates showed an EEG response that appeared characteristic for PDE. This specific child had previously been diagnosed with PDE, until normal urinary $\alpha$-AASA levels and absence of *ALDH7A1* gene mutation excluded the diagnosis. All together, during pyridoxine-IV, visual EEG analysis of the EEG-response to pyridoxine-IV can neither identify nor exclude PDE. Regarding the quantitative EEG responses, we observed a trend consisting of larger median % amplitude and % total power declines in PDE than in non-PDE infants. However, due to the large inter-individual overlap in outcomes, individual identification of neonatal PDE among non-PDE is prohibited. One of the strengths of the present study is that five of six PDE neonates were diagnosed with the same homogeneous genotype. Therefore, we cannot attribute the large inter-individual variability in EEG responses to different phenotype-genotype relationships. With the presently available data, one may only speculate whether the same results would have been expected in other affected neonates with different genotypes. Although this question has to be carefully addressed in a later study, the large inter-individual overlap between pyridoxine-IV EEG responses in neonates with *ALDH7A1* gene mutations appears suggestive for a non-specific effect, i.e. regardless of the genotype. Given the preceding, we hypothesize that the EEG response to pyridoxine-IV is rather dependent upon other, non-specific, factors such as medication, gestational age, encephalopathy, comorbidities, and factors other than on the genotype itself.

We are aware that the small number of PDE patients may provide a limitation to this study. However, since PDE is a rare condition, we included all six neonates (derived from the Dutch PDE cohort) with an available pyridoxine-IV EEG trial. Despite this relatively small number of patients, large inter-individual variations in EEG responses already hampered identification of PDE among non-PDE.



In conclusion, present data show that pyridoxine-IV causes non-specific EEG responses that neither identify nor exclude PDE caused by *ALDH7A1* gene mutations. These data implicate that neonates with TRS should continue to receive pyridoxine until PDE is fully excluded by biochemical and/or genetic analysis.

**Acknowledgements**
The authors would like to acknowledge Lucas Dijck for his help to prepare digital EEGs for analysis and Saskia Houterman for statistical advice.

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# Chapter III.b

## Antenatal treatment in two Dutch families with pyridoxine-dependent seizures



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Eur J Pediatr 2010 Mar; 1969(3):297-303

## Abstract

Incidental reports suggest that antenatal treatment of pyridoxine dependent seizures (PDS) may improve neurodevelopmental outcome of affected patients. Two families with PDS are reported, both with two affected siblings. Antenatal treatment with pyridoxine was instituted during the second pregnancy in each family (50 and 60 mg daily from 3 and 10 weeks of gestation, respectively). Perinatal characteristics and neurodevelopmental outcome at 4 (Family A) and 12 (Family B) years of age, were compared between the untreated and treated child within each family. Meconium- stained amniotic fluid was present in both first pregnancies and abnormal foetal movements were noticed in one. In the treated infants, pregnancy and birth were uncomplicated. In family A, postnatal pyridoxine supplementation prevented neonatal seizures. Both children in family A were hypotonic and started walking after 2 years of age; both had white matter changes on MRI, and the first child was treated for squint. IQ was 73 and 98 in the antenatally untreated and treated child, respectively. The second child in family B developed seizures on the seventh day, because pyridoxine maintenance therapy had not been instituted after birth. Seizures responded rapidly to pyridoxine supplementation. MRI showed large ventricles and a mega cisterna magna. IQ was 80 and 106 in the antenatally untreated and treated child respectively. Both children had normal motor development. These results suggest that antenatal pyridoxine supplementation may be effective in preventing intrauterine seizures, decreasing the risk of complicated birth, and improving neurodevelopmental outcome in PDS.

**Keywords:** Seizures, Pyridoxine, Antenatal treatment, Development.

## Introduction

Pyridoxine-dependent seizures (PDS; MIM 266100) is a rare autosomal recessive disorder with an estimated birth incidence between 1 in 396 000[1] and 1 in 783 000[2]. PDS has been recognised for over 50 years, since the first report by Hunt et al. in 1954[3]. The classic presentation of PDS consists of neonatal seizures that are intractable to conventional anti-epileptic drugs but adequately respond to pyridoxine (vitamin B6) administration. In retrospect, about 20% of mothers report abnormal foetal movements highly suggestive of intrauterine seizures[4]. Birth can be complicated, as a third of the children present with asphyxia and/or suspected hypoxic-ischaemic encephalopathy[2,4]. Not only is PDS characterised by seizures but also by encephalopathic symptoms such as agitation, jitteriness, irritability, startle reactions and feeding problems[4]. Reports on cerebral imaging have shown cerebral haemorrhage, non-specific white matter abnormalities, hydrocephalus, hypoplasia of the posterior part of the corpus callosum, cerebellar hypoplasia and a megacisterna magna[5-8]. At older age, cortical atrophy with ventricular dilation is sometimes observed in affected patients[5,6,9].

Until recently, PDS was a clinical diagnosis based upon effective seizure control by pyridoxine administration. In 2006, mutations in the *ALDH7A1* gene were shown to be present in the majority of patients with a clinical diagnosis of PDS[10-13]. *ALDH7A1* encodes the enzyme α–aminoadipic semialdehyde (α-AASA) dehydrogenase that plays a role in the degradation of the amino acid lysine. α-AASA dehydrogenase deficiency leads to increased levels of α-AASA in urine and plasma[11] and α-AASA can therefore be used as a biomarker for PDS[14]. There are at least two pathophysiological mechanisms that play a role in PDS. First, semialdehydes easily bind to many different molecules and accumulation of α-AASA might therefore have toxic effects on the (developing) central nervous system. Second, α-AASA is non-enzymatically converted to L-delta(1)-piperideine-6-carboxylate, which is a metabolite that binds pyridoxal-5-phosphate (the active B6 vitamer) and as such leads to secondary pyridoxal-5-phosphate deficiency[11].

Despite successful seizure control with pyridoxine substitution, the majority of surviving PDS patients show some degree of cognitive impairment, particularly concerning expressive language development. Only around 20% of treated PDS patients are described as having a normal development[4,15]. Reports of a possible relationship between treatment delay and cognitive impairment have been inconsistent[1,16]. Incidental reports of antenatal treatment with pyridoxine in a second pregnancy suggest that this approach may be effective in controlling intrauterine seizures[17] and may improve developmental outcome[3,17-22]. However, only eight cases of antenatal pyridoxine supplementation have been described, and with limited follow-up. Furthermore, treatment sometimes only coincidentally aimed for multi-vitamin supplementation in low doses and for a limited number of months. The importance of long-term follow-up is illustrated by reports of PDS patients showing an average development initially, though not based on a formal assessment, but experiencing developmental delays at a later stage[16,23,24].

We report two PDS families from the Dutch PDS cohort with long-term follow-up after antenatal treatment in a second pregnancy.

## Methods and patients

Currently the Dutch PDS cohort comprises 14 living children (born 1991 - 2007), including three families with two PDS patients each. In two of these families, the mother used pyridoxine daily during the second pregnancy from the first trimester onwards. Methodology and results of α-AASA measurements and *ALDH7A1* mutation analysis have previously been reported[13,14]. Table I presents patient data on the clinical course, imaging and outcome.

### Case Reports

**Family A, Child 1**. A girl was born at term with meconium-stained amniotic fluid and Apgar scores of 7 and 8 after 1 and 5 minutes, respectively. She developed clinical seizures within 1 hour after birth. Initially, seizures were temporarily controlled by phenobarbitone and clonazepam. Pyridoxine 50 mg was administered intravenously when seizures recurred on the 4$^{th}$ day of life; after this, seizures ceased within 5 minutes. Subsequently, the girl became hypotonic and needed respiratory support for 2 days. All anti-epileptic drugs were discontinued after administration of pyridoxine, and no maintenance therapy with pyridoxine was instituted. Seven days after the initial administration of pyridoxine, generalised seizures recurred. Again, seizures responded to parenteral administration of pyridoxine. The girl was diagnosed with probable PDS (patient 10[1]) according to the clinical criteria of Baxter[2] and maintenance monotherapy with pyridoxine 15 mg/kg/day was instituted. In retrospect the mother reported abnormal jerky foetal movements highly suggestive of intra-uterine seizures. MRI of the brain on the fifth day of life showed multiple bilateral lesions in the white matter on T2, corresponding with cerebral oedema. Diffusion-weighted (DW) images showed in general an increased apparent diffusion coefficient (ADC) with numerous focal lesions with a decreased ADC, corresponding with a structural abnormal cerebral white matter and recent cytotoxic lesions containing increased extracellular water. At the age of 5 months a second MRI showed a thin corpus callosum and slight asymmetric ventriculomegaly without clear white matter abnormalities. At 3 years of age (in 2006) the diagnosis of PDS was proven at the metabolic and DNA level (Table I). At the time of this report the child is 5 years of age and seizure-free on pyridoxine monotherapy. She is hypotonic and has motor dyspraxia. In addition, she was treated for squint and did not walk until the age of 2.75 years. Mental development according to the Snijders-Oomen Nonverbal Intelligence Test (SON-R)[25] is below average, particularly in expressive language skills (Table I). For these reasons, she currently attends a school for special education.



**Family A, Child 2**. During the second pregnancy of a son, the mother used pyridoxine 50 mg/day from the third week of gestation. Pregnancy and birth, as well as the neonatal period, were uneventful. From the first day of life pyridoxine supplementation 50 mg/day was instituted which was increased to 150 mg/day at 6 months of age. In the first year of life no seizures were noticed; at age 1 year the child experienced a febrile convulsion twice. At 1.5 years of age (in 2006) PDS was proven at the metabolic and DNA level (Table I). At the age of 4 years, he had a right-sided hemi status epilepticus during a febrile period with diarrhoea, which was controlled after 200 mg pyridoxine administered intravenously. MRI of the brain at age 1.5 year showed bilateral white matter abnormalities and a thin genu corpus callosum. He started walking at age 2¼ years. At the present age of 4 years, he is slightly hypotonic. His mental development, assessed using the SON-R, is average,

although there is a delay in expressive language skills, needing extra support. His MRI still shows circumscript bilateral frontal white matter abnormalities. The parents have no other children and have an average social economic status.

**Family B, Child 1**. The first child in this family, a girl, was born with meconium-stained amniotic fluid. She developed seizures 2 hours after birth. Pyridoxine 60 mg/day was started after the 10$^{th}$ week of life when trials with several anti-epileptic drugs had failed to control her seizures[26]. Ultrasound and CT scan of the brain showed no cerebral abnormalities, except for a small haematoma of the tentorium cerebelli. A trial of withdrawal was never performed and at 12 years of age PDS was proven at the metabolic and DNA level (Table I). At the moment of this report, the child is 14 years old, attends a regular school and has a normal motor development. A recent MRI of her brain showed no abnormalities.

**Family B, Child 2**. The mother used 60 mg pyridoxine daily during her second pregnancy from the 10$^{th}$ week of gestation onwards. Pregnancy and birth were uncomplicated. Erroneously no pyridoxine maintenance therapy was instituted after birth. The boy developed seizures on the seventh day of life, after which treatment was started with 60 mg pyridoxine daily. Consequently seizures were controlled. At 10 years of age PDS was proven at the metabolic and DNA level (Table I). The boy is currently doing well and shows no abnormalities on neurological examination; he attends a regular school and has a normal motor development. MRI of the brain at age 12 years showed slightly enlarged lateral ventricles and some enlargement of the cisterna magna with a normal corpus callosum. Table I presents data on the mental development of these children using the WISC-III[27]. The parents have no other children and have an average social economic status.

## Discussion

In this report on antenatal treatment in PDS, the first child was regarded as the 'natural' control patient of the antenatally treated second PDS patient in that same family. Pyridoxine was well tolerated by the mother and no abnormal foetal movements were reported in the antenatally treated PDS patients. Moreover, pregnancy, labour and birth were uncomplicated after antenatal treatment with pyridoxine. These observations suggest that antenatal treatment of PDS may be an effective treatment to prevent intrauterine seizures and reduce birth-related complications[4].



Within each family the same intelligence test was used to compare the sibs. In family A, a non-verbal developmental test (SON-R) was used because this test has sound psychometric properties and is often used for children with speech problems, as was the case for these children. In the second family the WISC-III was used, which measures both verbal and non-verbal capacities and is often used to determine intellectual capabilities of children attending regular education. The antenatally treated children in both families were shown to have better test results than their first-born sibs. This suggests that antenatal pyridoxine treatment may improve mental development, although some degree of developmental delay remained in the second child of family A. We were not able to compare the outcomes

**Table 1.** Clinical characteristics and development outcome of antenatal treatment

| | **Family A** 1st Child | **Family A** 2nd Child | **Family B** 1st Child | **Family B** 2nd Child |
|---|---|---|---|---|
| **Antenatal** | | | | |
| Pyridoxine dose | No | 50 mg, 3rd gestational w | No | 60 mg, 10th gestational w |
| **Perinatal** | | | | |
| Intra-uterine movements | Abnormal ('earthquakes') | Normal | Normal | Normal |
| Birth, APGAR score | Meconium, 7 and 8 | 9 and 10 | Meconium, 7 and 9 | 9 and 10 |
| **Diagnosis** | | | | |
| Age at onset seizures | 1 hour | None | 2 hour | 7 days |
| AASA urine * | 40 | 75 | 28 | 20 |
| DNA mutation | c.[1195 G > C]+ [1195G > C] | c.[1195 G > C]+ [1195G > C] | c.[1195 G > C]+ [1195G > C] | c.[1195 G > C]+ [1195G > C] |
| **Treatment** | | | | |
| Start B6 | 4th day of life | Antenatal 50 mg, continued after birth | 10th week of life | Antenatal 60 mg. Discontinued after birth, restarted after 7d day of life |
| Current Pyridoxine dose | 150 (5 - 10 mg/kg/day) | 150 (5 - 10 mg/kg/day) | 60 mg/day | 60 mg/day |
| **Outcome** | | | | |
| Test Age (yr) | 5 | 4 | 14 | 12 |
| Head circumference | + 1 SD | + 1 SD | + 2 SD | + 4 SD |
| MRI (recent) | WM, CCH, LV. Neonatal WM ++, diffusion | WM + FL + CCH | Normal (Neonatal CT: small haematoma tentorium cerebelli) | LV + cisterna magma |
| Total IQ | SON-R: 73 | SON-R: 98 | WISC-III: 80 | WISC-III: 106 |
| | SON Performal: 80 | SON Performal: 90 | WISC Performal: 76 | WISC Performal: 96 |
| | SON Reasoning: 72 | SON Reasoning: 109 | WISC Verbal: 87 | WISC Verbal: 114 |
| Motor performance | Hypotonic, walking 31 m | Hypotonic, walking 27 m | Normal | Normal |
| School | Special Education | Regular education + extra support | Regular education | Regular education |

AT = antenatal treatment, Apgar was scored at 1 and 5 minutes, meconium = meconium-stained amniotic fluid, LV = large ventricles, WM = white matter abnormalities, FL = focal lesions, CCH = corpus callosum hypoplasia, w = week, m = month, *AASA urine in mmol/mol creatinine, Normal < 1 mmol/mol creatinine

**Table II.** Patients in the present study compared with published data on antenatal treatment

| Author | Hunt[3] | Scriver[22] | Bejsov[17] | Iinuma[18] | Pettit[20] | Nabbout[19] | Rankin[21] (n = 2) | Patient A2 | Patient B2 |
|---|---|---|---|---|---|---|---|---|---|
| Year of publication | 1954 | 1960 | 1967 | 1970 | 1987 | 199[a] | 2007[a] | | |
| B6 dose pregnancy mg/d | 20 - 30 | 9 - 12[b] | 90 - 110[c] | 12 | 4 | Unknown | 250 | 50 | 60 |
| Reason for maternal B6 | M V | M V | AT | M V | M V | AT | AT | AT | AT |
| Metabolic/DNA PDS | - | - | - | - | - | - | + | + | + |
| Period during pregnancy | 2 – 5 m | 3 – 9 m | 8 – 9 m | 5 – 9 m | 3 – 9 m | 0 – 9 m | 0 – 9 m | 3 w - 9 m | 10 w - 9 m |
| B6 dose after birth | 6 mg/5d | 1 - 10 mg | 25 mg | 30 mg | 15 mg | Unknown | 15 mg/kg/d | 50 mg | 60 mg |
| Started at age of | 23 d | 25 d | Birth | 4 m | 2 d | Birth | Birth | Birth | 1 w |
| Follow-up age in report | 9 y[36] | 15 m | 6 m | 15 m | 4 m | 1 y | 10 & 8 y | 4 y | 12 y |
| Short-term follow-up | Retarded | Normal | Normal | Retarded | Normal | Normal | Unknown | Sub-normal | Normal |
| Long-term follow-up | IQ 35 | Normal | Some Delay | Unknown | Unknown | IQ 60 | IQ 54 & 52 | IQ 98 | IQ 106 |

MV = multivitamin, AT = antenatal treatment, d = day, w = week, m = month, y = year, [a] = Possibly the same family from France including 3 siblings with PDS, [b] = 9 mg for the second and 12 mg for the third trimester, [c] = 90 mg initially increased to 110 mg after 2 days.

of the affected children to healthy non-affected siblings, since there were none for both families. All four parents have attended regular education.

Interestingly, in family B the first child developed clinical seizures 2 hours after birth, whereas the second child developed clinical seizures on the seventh day of life. In children with PDS who stop pyridoxine treatment, seizure recurrence is usually reported within 5 - 7 days[4,16]; suggesting a pyridoxine 'storage' sufficient to temporarily prevent seizure occurrence in these patients. These observations further suggest that in the present study, the second child was adequately treated antenatally and that the pyridoxine dose prevented seizures shortly after birth. Conversely, seizure occurrence on day 7 could also reflect the different effect of withdrawal in neonates in whom seizure recurrence can be delayed by up to 6 weeks[16]. This observation might indicate what the optimum pyridoxine dose might be during pregnancy. Hyperemesis gravidarum is treated with 50 - 100 mg pyridoxine a day, which is well tolerated by both mother and foetus[16]. Others suggested a necessarily daily supplement of 2.5 to 10 mg pyridoxine in all pregnant women and have shown that the pyridoxine state of the mother significantly affects the foetus[28,29]. In general, only long term use of pyridoxine in doses over 200 - 300 mg a day is thought to lead to advers effects, especially reversible sensory neuropathy in healthy adults[30]. Based on these observations we suggest that a daily dose of 50 mg is safe for the mother and may prevent foetal seizures, complications at birth, and early neonatal seizures in PDS patients.

In the present study, MRI of the brain showed abnormalities in 3 of the 4 reported children, including in the antenatally treated children. This observation is important and suggests that antenatal pyridoxine treatment does not guarantee normal brain development and that additional mechanisms (besides seizure-induced injury) may be involved in PDS patients. These may include either toxicity due to elevated levels of $\alpha$-AASA or the lack of metabolite(s) after the metabolic block, both of which may cause neuronal damage/changes irrespective of pyridoxine treatment. In that case, interventions to prevent or treat these metabolic changes in PDS patients should be investigated in order to improve future outcome in this group. The disappearance of the neonatal white matters abnormalities seen in patient 1 (Family A) has been interpreted by us as the result of seizure control.



This is one of the few reports focusing on antenatal treatment of anticipated/possible PDS in a second child, including long-term and case-controlled follow-up. Until now, antenatal treatment of PDS has been reported in only 8 patients[3,17-22] and two of these reports (regarding a family that moved from France to England) might overlap[19,21]. The reports are not uniform regarding the daily antenatal doses, the period of antenatal treatment, the reason for treatment, or the reported (side)effects (Table II). No complications at birth and no neonatal seizures in the first week of life were reported for any of the treated patients. Only four patients were formally tested regarding development outcome, all of whom showed some degree of developmental delay. In the remaining four patients, developmental outcome was either roughly described as 'normal' (n = 3) or 'delayed' (n = 1). Only one report mentioned a control PDS sib who did not receive antenatal pyridoxine treatment; this report concluded that antenatal treatment does not improve outcome, although the antenatally treated children scored about 10 IQ points higher than the PDS patient that did not receive antenatal pyridoxine[21].

Because of the autosomal recessive nature of PDS, the recurrence risk is 25% in consecutive pregnancies. The metabolic and genetic defect of PDS has recently been elucidated[11], which gives the opportunity for prenatal diagnostic investigation. In selected families prenatal diagnosis and/or antenatal pyridoxine treatment can be offered. Antenatal pyridoxine treatment should start as early as possible in pregnancy, and not be postponed until the results of eventual prenatal diagnostic tests are available.

In both families, the affected children were homozygous for the – common – c.[1195G > C]; mutation, which has a relatively high frequency in the Netherlands (15 of 20 alleles[13]), and has also been frequently identified by others (12 of 36 alleles[12]) (11 of 30 alleles[31]). The mutation leads to substitution of glutamate at position 399 by glutamine p[Glu399Gln] and is predicted to have a negatively effect on the binding capacity of $\alpha$-AASA dehydrogenase to the required cofactor $NAD^+$, resulting in decreased enzyme activity[32].

Intrauterine treatment of a foetus that suffers from a genetic metabolic disorder is rarely possible. Our report shows the possible positive effects that can be reached, and it aims to increase the awareness of clinicians regarding this 'unusual' treatment modality. Besides PDS, there are at least three other autosomal recessively inherited metabolic disorders that are good candidates for antenatal treatment. In 3-phosphoglycerate-dehydrogenase deficiency (a disorder of serine biosynthesis) supplementation of L-serine has been successful[33]. Vitamin B12-responsive methylmalonic aciduria can be treated by supplementation of the mother with high doses of vitamin B12[34]. Finally, another defect of pyridoxine metabolism, namely pyridox(am)ine-5'-phosphate oxidase, should (at least theoretically) be treatable by supplementation of pyridoxal-5'-phosphate, the active form of pyridoxine[35]. The current study is limited by its retrospective nature and the small sample size, which is due to the rare nature of PDS. However all previous reports on antenatal treatment are case reports, and most do not describe control patients. Prospective studies on the effects of antenatal treatment of PDS should be performed.

We conclude that, after a previous child with PDS, there are strong arguments to supply mothers with pyridoxine during subsequent pregnancies. Using this approach, foetal and neonatal seizures and birth-related complications in affected sibs may be prevented. Finally, our data suggest that antenatal treatment may improve developmental outcome in subsequent PDS patients, compared to untreated siblings.

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# Long-term outcome in pyridoxine-dependent epilepsy



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Dev Med & Child Neurol 2012; sept 54(9):849-854

## Abstract

**Aim:** The long-term outcome of the Dutch pyridoxine-dependent epilepsy cohort and correlations between patient characteristics and follow-up data were retrospectively studied.

**Method**: Fourteen patients recruited from a national reference laboratory were included (four males, 10 females, from 11 families; median age at assessment 6 years; range 2 year 6 months – 16 years). The following data were retrieved: sex; age at seizure onset; age at start pyridoxine therapy; level of urinary alpha-aminoadipic acid semialdehyde; antiquitin mutations; developmental milestones; evaluation of neurocognitive functioning and school career; magnetic resonance imaging (MRI) and electroencephalography (EEG) assessments.

**Results**: Pyridoxine was started antenatally in two children, in the first week of life in five, in the first month of life in three, or after the first month of life (range 2.5 – 8 months) in four. No children were physically disabled; however, only five walked at 2 years of age. Mental development was delayed in most: median IQ or developmental index was 72 (SD 19). Pyridoxine monotherapy controlled seizures in 10 of 14 children, whereas four needed additional antiepileptic drugs. Seizure persistence, antiepileptic drugs (other than pyridoxine), EEG background, and epileptiform activity were not associated with outcome. On neonatal MRI, structural and white matter abnormalities occurred in five of eight children; on follow-up, the number of abnormal MRI was increased. Delayed initiation of pyridoxine medication and corpus callosum abnormalities were significantly associated with unfavourable neurodevelopmental outcome, but normal follow-up imaging did not predict a good outcome.

**Interpretation**: Outcome of patients with pyridoxine-dependent epilepsy remains poor. Individual outcome cannot be predicted by the evaluated characteristics. We suggest that collaborated research in structured settings could help to improve treatment strategies and outcome for pyridoxine-dependent epilepsy.


### Abbreviations

| | |
|---|---|
| α-AASA | Alpha-aminoadipic acid semialdehyde |
| PDE | Pyridoxine-dependent epilepsy |
| WMA | White matter abnormalities |



### What this paper adds

- Seizure control was reached in all children with pyridoxine-dependent epilepsy, but necessitated the use of additional antiepileptic drugs in four.
- Age at start of treatment and corpus callosum abnormalities significantly correlated with outcome.
- Normal follow-up imaging did not predict good outcome.

## Introduction

Pyridoxine-dependent epilepsy (PDE) is a rare autosomal recessive disorder[1], with an incidence of approximately one in 400 000 children in the Netherlands[2]. The underlying metabolic defect in the cerebral lysine degradation pathway, alpha-aminoadipic acid semialdehyde (α-AASA) dehydrogenase (antiquitin) deficiency, results in accumulation of several metabolites, mainly α-AASA, $\Delta^1$-piperideine-6-carboxylate, and pipecolic acid, and secondary pyridoxine depletion of the central nervous system[3,4]. Treatment with pyridoxine is rational and generally sufficient to control the seizure disorder.

Despite the recent elucidation of the molecular basis for PDE[4] and the rapidly increasing number of reported patients since then, detailed data on long-term outcome remain scarce. Overall, outcome in PDE is often considered to be poor[5]. To contribute to the understanding of the prognosis of children with PDE, we studied the long-term outcome of a previously described cohort of patients with PDE in the Netherlands by standardized evaluation of present neurocognitive functioning, and systematic review of all available magnetic resonance imaging (MRI) and electroencephalography (EEG) studies. We studied possible relations between the acquired follow-up data and the initial patient characteristics at diagnosis (i.e. the age at diagnosis, age at start of pyridoxine supplementation, and genetic and biochemical data).

## Method

For this study, we included all patients with PDE (n = 14: four males, 10 females, from 11 families; median age at assessment 6 years; range 2 years 6 months - 16 years) diagnosed in our (nationwide reference) laboratory and born in the Netherlands between 1991 and 2008 (Table I). Three families had two affected children; in these families a diagnosis of PDE in the first born was made before the birth of the second affected child. In two families the mother started daily pyridoxine in the first trimester of her second pregnancy, 60 mg (patient 1) and 50 mg respectively (Bok et al.[6]). PDE was proven in all patients by demonstration of increased urinary α-AASA concentrations and mutations in the antiquitin gene[3,7]. α-AASA in urine was measured by liquid chromatography-tandem mass spectrometry as previously published[4]. The following data were retrieved from the medical records of all patients: sex; age at seizure onset and age at start of pyridoxine therapy; urinary α-AASA level and antiquitin mutations at diagnosis; vitamin B6 intake (milligrams per kilogram); developmental milestones such as age at independent walking and school career (regular school or special education); and current medication.

All patients underwent a standardized neurocognitive test battery for this study (n = 6) or test results were retrieved from the records (n = 8). The Bayley Scales of Infant Development (2nd edition, Dutch manual) was used for children below 3 years of age[8], the Snijders-Oomen Non-Verbal Intelligence Test (Revised) 2.5 - 7 for children between 2.5 and 7 years of age[9], and the Wechsler Intelligence Scale for Children (3rd edition; WISC-III) for children between 8 and 17 years of age[10]. The Wechsler Intelligence Scale for Children also provides separate outcomes for performance and verbal IQ. These tests

have been judged as sufficient (Bayley Scales of Infant Development) or good (Snijders-Oomen Non-Verbal Intelligence Test and WICS-III); test results can be compared with one another[10]. Developmental outcome was defined as normal with total development index (for the Bayley Scales of Infant Development) or IQ (for WISC-III and Snijders-Oomen Non-Verbal Intelligence Test) scores above 85 (group 1), mildly delayed with total developmental outcome scores between 70 and 84 (group 2), moderately delayed with total developmental outcome scores between 55 and 69 (group 3), and severely delayed with total developmental outcome scores under 55 (group 4). This definition was used for this study and is based (groups 1 – 3) on the Bayley Scales of Infant Development with a subdivision of group 4 with severely delayed development[8]. SD was set at 15 IQ points. The median age at assessment was 6 years (range 2.5 – 16 years).

**Table I.** Characteristics of patients with pyridoxine-dependent epilepsy

| Patient | Male/ female | Seizure onset | Start B6 | α-AASA urine (mmol/mol creatinine) | DNA mutation |
|---|---|---|---|---|---|
| 1[a] | M | Not | Antenatal | 20 | c.[1195 G > C]+[1195G > C] |
| 2[b] | M | Not | Antenatal | 75 | c.[1195 G > C]+[1195G > C] |
| 3 | F | 1 d | 16 d | 32 | c.[1195 G > C]+[1195G > C] |
| 4 | F | 5 d | 6 mo | 5 | c.[750G > A]+[1348T > A] |
| 5[a] | F | 1 d | 2.5 m | 29 | c.[1195 G > C]+[1195G > C] |
| 6[c] | F | 2 d | 10 d | 16 | c.[1195 G > C]+[1195G > C] |
| 7[b] | F | Intrauterine | 5 d | 39 | c.[1195 G > C]+[1195G > C] |
| 8 | F | 3 d | 2.5 mo | 4 | c.[750G > A]+[750G > A] |
| 9 | F | 2 d | 5 d | 99 | c.[1195 G > C]+[1195G > C] |
| 10 | M | 2 d | 13 d | 60 | c.[1195 G > C]+[1195G > C] |
| 11 | F | 0 d | 3 d | 71 | c.[1195 G > C]+[1195G > C] |
| 12[c] | F | 2 d | 3 d | 24 | c.[1195 G > C]+[1195G > C] |
| 13 | F | 1 d | 8 mo | 10 | c.[1195 G > C]+[1195G > C] |
| 14 | M | 1 d | 3 d | 12 | c.[1195 G > C]+[244C > T] |

M, male; F, female; α-AASA, alpha-aminoadipic acid semialdehyde; [a,b,c]pair of sibs (1 and 5; 2 and 7; 6 and 12); reference ranges for urinary α-AASA: ≤ 6 months, < 2 mmol/mol creatinine; > 6 months to 1 year, ≤ 1 mmol/mol creatinine; > 1 year, ≤ 0.5 mmol/mol creatinine

We collected all available MRI and EEG recordings of the 14 children with PDE. As MRIs and EEGs were collected over a period of 15 years from eight different Dutch centres, different machines and protocols had been used for MRI and EEG recordings. Although most of the MRIs were retrospectively collected and not done for this study, most MRI studies were extensive. Minimally studied MRI sequences included axial T1 and T2 and sagittal T1 images. All MRIs had been evaluated by a neuroradiologist in the original centre and were

reviewed and scored separately by two investigators (LAB and MAW). A structured method was used by the investigators to study all MRIs.[11] A mega cisterna magnum was defined as a distance of more than 10 mm, and an enlarged cisterna magnum as a distance of 5 to 10 mm, between cerebellum and skull. All EEGs were qualitatively assessed by two investigators (DAS and JHvdH). We applied the term 'epileptiform' activity for interictal sharp waves (i.e. peaked configurations at paper speed 70 – 200 ms, observable in theta and/or delta frequency bands). We reserved the term 'discrete' epileptiform activity to denote sporadic, isolated sharp waves at a frequency of fewer than 10 per 30 minutes.

The study was approved by the institutional review board of Màxima Medical Centre, Veldhoven, the Netherlands. Signed, informed parental consent was obtained to collect the studied data.

### Statistical analysis

A Mann–Whitney *U* test and Fisher's exact test were used to analyse non-parametric divided numeric and categorical data respectively. Significance was defined as $p < 0.05$. Spearman's correlation coefficients were estimated.

## Results

### Neurodevelopmental outcome

Test results are summarized in table II. All 14 children had learned to walk independently (mean age 26 months); however, only five children could walk independently before the age of 2 years. No child was physically disabled and all of the children were in level I of the Gross Motor Function Classification Score. Six children attended regular school; three of these children needed extra support at school. Eight children attended a school for special education. One child had been treated for hydrocephalus by third ventriculostomy.

Normal cognitive development (IQ or developmental index > 85) was seen in four children, mildly delayed development (IQ or developmental index 70 - 84) in four children, moderately delayed development (IQ or developmental index 55 - 69) in three children, and severely retarded development (IQ or developmental index < 55) also in three children. Mean IQ was 72 (SD 19; median IQ 72). Performance versus verbal IQ was tested in five children. In four of these children, verbal IQ was higher than performance IQ. In only one patient did this difference reached significance of more than one SD. In six children between 3 and 7 years of age a pretest assumption of poorly developed Verbal skills was made by the psychologist, and subsequently a developmental test, the Snijders-Oomen Non-verbal Intelligence Test, was chosen. This implied that comparison between Verbal and Performance IQ could not be studied in these children. Although in most reports the psychologist made some remarks about the behaviour of the studied child, we did not perform a structured systematic behavioural study in the children with PDE. The remarks on behaviour were normal behaviour (n = 9), good concentration (n = 10), not well concentrated (n = 2), dyspraxia (n = 3), timid (n = 2), dysphasia (n = 1), unknown (n = 2).

**Table II.** Developmental outcome and imaging of patients with pyridoxine-dependent epilepsy

| Patient | Development | | | | | MRI | |
|---|---|---|---|---|---|---|---|
| | Age at walking (y) | School | Test | Age at test (y) | IQ (P/V) or DI | Neonatal | Follow-up |
| 1[a] | 1.3 | R | WISC | 12 | 108 (96/114) | NA | VM eCM |
| 2[b] | 2.3 | R+ | SON-R | 3 | 102 | NA | CCH WMA VM |
| 3 | 2 | S | BSID-II | 3.5 | 87 | WMA[d] | WMA VM |
| 4 | 1.8 | R | WISC | 16 | 86 (84/91) | NA | Normal |
| 5[a] | 1.5 | R | WISC | 15 | 80 (76/87) | Haemorrhage | Normal |
| 6[c] | 2 | R+ | WISC | 12 | 77 (78/80) | NA | WMA, eCM |
| 7[b] | 2.8 | S | SON-R | 5 | 73 | CCH WMA[d] | NA |
| 8 | 1.9 | R+ | WISC | 16 | 71 (76/72) | NA | Normal |
| 9 | 1.5 | S | BSID-II | 2.5 | 63 | WMA[d] | Cyst |
| 10 | 2.5 | S | BSID-II | 2.5 | 57 | CCD eCM WMA[d] | CCH WMA |
| 11 | 2 | S | SON-R | 5 | 55 | Haemorrhage (on CT) | CCD |
| 12[c] | 3.5 | S | SON-R | 7 | 50 | CCD WMA | CCD WMA VM |
| 13 | 3 | S | SON-R | 5 | 54 | Haemorrhage | CCH VM |
| 14 | 2.4 | S | SON-R | 7 | 50 | Normal | CCH VM MCM |

[a,b,c] Pair of sibs (1 and 5; 2 and 7; 6 and 12); MRI, magnetic resonance imaging; P/V, performance IQ versus verbal IQ after Wechsler Intelligence Scale for Children (WISC) if applicable; DI, developmental index (Bayley Scales of Infant Development ($2^{nd}$ edition) [BSID-II]); NA , not available; VM, ventriculomegaly; eCM, enlarged cisterna magna; Son-R, Snijders-Oomen Non-Verbal Intelligence Test; CCH, corpus callosum hypoplasia; CCD, corpus callosum dysplasia; MCM, mega cisterna magna; WMA, white matter abnormalities on $T_2$; WMA[d], white matter abnormalities on diffusion imaging abnormalities and not on $T_2$; CT, computed tomography; R, regular education; R+, regular education with extra support; S, special education.

## Neuroimaging

### *Neonatal MRI*

Neonatal T1- and T2-weighted MR images were available for analysis for eight children; in four children additional diffusion-weighted images were available, and in one child we assessed neonatal CT images. Corpus callosum was normal in five children, dysplastic in two (splenium), and hypoplastic in one child. Neonatal intracranial, subdural haemorrhage in the vicinity of the falx and tentorium cerebri was observed in three children. On T1 and T2, no distinctive white matter abnormalities (WMA) were seen, except in one patient. However, diffusion-weighted images revealed abnormal signals in all four studies. Lesions with an increased apparent diffusion coefficient were predominantly located in the frontal and parietal regions. In two neonates, small focal lesions with decreased apparent diffusion coefficient, next to extensive regions with increased apparent diffusion coefficient, were seen.

### *Follow-up MRI*

In 13 children MRI could be studied after the neonatal period (range 3 months – 16 years of age; table II). Structural anomalies were still present on follow-up MRIs in all patients with neonatal MRI anomalies. One patient had a mega cisterna magnum on follow-up that was not observed at the time of neonatal imaging; this was the patient who required shunting. Taking all radiologic data together, the corpus callosum was normal in seven children, four had a hypoplastic posterior part (isthmus) and three had an abnormal dysplastic posterior part (splenium) of the corpus callosum. Ventriculomegaly was observed in six children. Four had a normal ventricular size on their neonatal MRI. In the other two children, neonatal MRI was unavailable. In three children with ventriculomegaly, MRI suggested the presence of a narrow aqueduct.

In five children, WMA were present on T2-weighted imaging as discrete, multifocal lesions (predominantly) in the periventricular and/or central zone. WMA were located at frontal (n = 3), parietal (n = 1), or occipital zones (n = 1). On follow-up, diffusion-weighted images and apparent diffusion coefficient were normal in all children. A mega cisterna magnum was seen in one child. An enlarged cisterna magnum was seen in three children.

### *Focal signal abnormalities*

An abnormal focal MRI signal was seen in the globus pallidus (hypointense on T1) in one child, in a cerebellar peduncle (hyperintense on T2; Fig. I) in two children, and in the frontal subcortical white matter in one child (hyperintense on T2; Fig. I). In this last child there was a slight increase in size over time. A unilateral periventricular cyst (4 mm) just above the frontal horn was seen in one child, and a bone cyst was observed in the orbital bone of another.



## EEG characteristics

Neonatal EEG characteristics have been described previously[12]. At follow-up, EEG background activity had normalized in 12 of 14 children (Table III). Only in one patient did we observe polymorphic spike wave-like activity (child 3). In five children epileptiform activity was absent, in five it was only discrete, and in four it appeared more pronounced. Epileptiform activity was located at frontal (in five children), fronto-central (in three), temporal (in one), or central (in one) regions.

**Figure I.** Axial fluid-attenuated inversion recovery image of patient 2 showing bifrontal subcortical hyperintense signal abnormalities

### Patient characteristics in relation to developmental outcome

***Start of treatment***

Children treated antenatally had a higher IQ than their siblings and had the highest IQ scores in group 1. Outcome of antenatal treatment versus postnatal treatment was significant in this group. Of the eight children who were treated in the first month of life, five revealed a developmental outcome in group 1 or 2, and three in group 3 or 4. Of the five children who were treated after 2.5 months of age, two showed a developmental outcome in group 1 or 2, and three in group 3 or 4. One patient who was only treated after 6 months of age had severely delayed development. Although there was no significant correlation between long-term neurodevelopmental outcome and age at which treatment was initiated, we observed a suggestive trend in favour of early treatment initiation. No pyridoxine daily doses were changed after 1 year of age.

**Table III**. EEG characteristics of patients with pyridoxine-dependent epilepsy

| Patient | Therapy | | | EEG characteristics | | |
|---|---|---|---|---|---|---|
| | Age | B6 | AED | Background activity for age | Epileptiform activity | Seizure type |
| 1[a] | 12 y | | None | Slowed | θ (F) | — |
| 2[b] | 2 y | 1.2 | CBZ | Normal | Absent | I, II |
| 3 | 1.5 mo | 6 | VPA | Normal | Δ (F - C) | I |
| 4 | 15 y | 1.8 | None | Normal | Sporadic θ (F) | — |
| 5[a] | 15 y | 1 | None | Normal | θ (F) | — |
| 6[c] | 14 y | 1 | None | Normal | Sporadic θ (F and T) | — |
| 7[b] | 4 y | 9 | None | Normal | Absent | — |
| 8 | 15 y | 1.3 | None | Normal | Absent | — |
| 9 | 2 y | 7.5 | PHB | Normal | Sporadic Δ (C) | I, III |
| 10 | 1 mo | 15 | None | Normal | Δ (F - C) | — |
| 11 | 3 y | 4.8 | VPA | Normal | θ (F - C) | I |
| 12[c] | 14 y | 1 | None | Intermittently slowed | Absent | — |
| 13 | 5 y | 3 | None | Normal | Sporadic Δ (F) | — |
| 14 | 3 y | 9 | None | Normal | Absent | — |

All electroencephalograms (EEGs) revealed continuous and reactive brain activity; [a,b,c]pair of sibs (1 and 5; 2 and 7; 6 and 12); seizure types: —, none; I, febrile seizures; II, partial seizures; III, generalized seizures; B6, daily vitamin B6 dose in mg/kg body weight; AED, antiepileptic drug; CBZ, carbamazepine; VPA, valproic acid; PHB, phenobarbital; θ, epileptiform activity in theta spectrum; Δ, epileptiform activity in delta spectrum; (C), at central region; (T), at temporal region; (F), at frontal region; (F - C), at fronto-central region.

***Genotype outcome***
Three children had a different mutation than the common Dutch one (Table I): two had a developmental outcome in groups 1 or 2, the other in group 4 (not significant).

***Seizure onset, persistence of epilepsy or EEG abnormalities, use of antiepileptic drugs***
Because all PDE seizures had started during the first few days of life, we did not observe a relation between seizure onset and developmental outcome (Table III). The persistence of seizures, the use of antiepileptic drugs (other than pyridoxine), the EEG background activity, and presence of epileptiform activity appeared to be unassociated with outcome.

***MRI and developmental outcome***
Of the seven children with an abnormal corpus callosum, five had a development outcome in group 3 or 4, whereas of the seven children with a normal corpus callosum only one child had a development outcome in group 3 (abnormal or normal corpus callosum versus IQ or developmental index less or more than 70; significant). No data were available to determine an association between corpus callosum abnormalities and Verbal or Performance IQ.

***Other parameters and developmental outcome***
There was also no statistical relation between outcome and urinary $\alpha$-AASA levels at presentation, outcome and daily pyridoxine dose, or outcome and head circumference. Intellectual outcome was related to age at independent walking.

## Discussion

In the Dutch PDE cohort, we aimed to elucidate the underlying relationship between clinical, radiological, electrophysiological, and biochemical parameters in developmental perspective. In this present cohort, all children had the classic phenotype of PDE with seizure onset shortly after birth, and most patients shared the same genotype. None of these children suffered from severe motor disturbances and all had learned to walk independently. The individual courses of intellectual development showed a wide range and most patients suffered from a delayed development, which is in accordance with the literature. In 10 of 14 children, seizures were well controlled with monotherapy pyridoxine, and four of 14 children received long-term treatment with additional antiepileptic drugs, also reported by others[13,14]. Outcome was significantly correlated with corpus callosum abnormalities and antenatal start of pyridoxine treatment.



A relatively high prevalence of structural anomalies and WMA was seen on neonatal MRI, including one antenatal treated patient, which raises the question whether structural MRI abnormalities should be explained as an intrinsic part of the phenotype or as phenomena that are secondary to metabolic disturbances or treatment failure. Although corpus callosum abnormalities appeared associated with unfavourable neurodevelopmental outcome, a normal MRI at follow-up did not warrant good outcome.

So far, cerebral MRI characteristics have been described in 50 patients[4,15–18]. In these

patients, structural anomalies like corpus callosum hypoplasia, mega cisterna magnum, as well as non-specific multifocal T2 WMA, cerebral atrophy, and hydrocephalus, are common findings. However, a normal MRI does not exclude PDE. In accordance with the literature, we also observed frequent MRI anomalies, involving structural corpus callosum abnormalities in half of the studied children. Although corpus callosum abnormalities have been described before (especially a thin posterior part[15]), the dysplastic shortening of the corpus callosum is a new finding.

Although the presence of ventriculomegaly in PDE has been reported incidentally, no data exist on its prevalence. In this cohort it was seen at follow-up in six of the studied children. Interestingly, ventriculomegaly was present in a neonate who had received prenatal treatment, and in some children ventriculomegaly developed after the neonatal period. In three of the six patients, ventriculomegaly coincided with an apparently narrow aqueduct, even necessitating shunting of the cerebrospinal fluid in one. Although these numbers are small, it appears advisory to monitor children with PDE for development of raised intracranial pressure.

In four out of nine neonates with PDE, we observed WMA (indicated by abnormal diffusion-weighted images with increased apparent diffusion-coefficient signal intensity). Interestingly these transient diffusion-weighted images/WMA, which are not uncommon in vasogenic edema, were mainly frontally located, whereas moderate to mild epileptiform activity was also frontally located. It remains speculative whether these morphological and functional abnormalities of frontal (white and grey) matter are associated and whether they should be regarded as common features of the underlying metabolic and epileptic encephalopathy.

In contrast to others[14], we were unable to demonstrate a relation between epileptiform activity, persisting seizures (including antiepileptic drugs) and neurodevelopmental outcome. This can be explained by the strong normalizing trend of follow-up EEGs, which have been described before[16,19,20]. The finding that early (postnatal) initiation of pyridoxine treatment does not sufficiently save the patient from long-term neurocognitive deficits, and the occurrence of different MRI abnormalities at different ages (i.e. at fetal [structural], neonatal [transient], and older age), may reflect the complexity of the underlying pathophysiological mechanisms of PDE[6].



The wide range of developmental outcome in this PDE cohort is in accordance with the literature. We have reviewed developmental outcome data of 93 patients with PDE that we found in the literature[4,15–17,20–26]. In those studies (five) that presented quantitative data on IQ[15,21,23,25,26] (a total of 24 patients), mean IQ was 68, comparable to our data. Using less detailed developmental scores for categorizing patients in whom exact data were lacking (i.e. normal versus mild, moderate, or severe delay) revealed similar results: normal development was seen in 25% (n = 24), mildly delayed in 27% (n = 25), moderately delayed in 24% (n = 22), and severely delayed development in 24% (n = 22).

Interesting results were found for the outcome of Verbal versus Performance IQ. In the normal population, Performance IQ equals Verbal IQ. In the literature, 17 of 24 patients

with PDE in whom Verbal and Performance IQ data were studied, Performance IQ was significantly higher than the Verbal IQ[15,21,23,25,26]. In the present study, Verbal IQ exceeded Performance IQ in the tested children. These test results do not support the findings of others[15] who found a poor development of expressive language skills. These discrepancies with PDE indicate that ongoing testing during development in each PDE patient is important.

Owing to the low prevalence of PDE, the studied cohort was small. This makes statistical analysis difficult and less sensible, and conclusions limited (low power). In addition, the retrospective character of this study, at least in the acquisition of most clinical, radiological, and EEG data, as well as disease course during the neonatal period and early development, precludes firm conclusions. Enlarging the number of patients, for example by combining cohort databases, and structured prospective follow-up will provide better insight in the clinical course of PDE, and its correlation with different clinical, metabolic, and radiological parameters. Only when we learn more about the long-term course of the disease will we be able to optimize already available treatment options (pyridoxine supplementation) and to develop novel (e.g. dietary) interventions.

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# Chapter III.d

## Roth spots in Pyridoxine Dependent Epilepsy

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BMJ-Case Reports 17 aug 2011

## Summary

Pyridoxine Dependent Epilepsy (PDE) is a rare metabolic[1] defect in the degradation of lysine. The authors report a patient with metabolic and DNA confirmed PDE, on the fifth day of life opthalmoscopy showed bilateral multiple white centered retinal haemorrhages, so called Roth Spots. Roth spots are non-specific haemorrhagic signs that occur in a variety of conditions of acute systemic insults in homeostasis - most often infections - which relate to retinal capillary damage and the ensuing reparative process. No biochemical or microbiological signs of infection were present in blood and cerebrospinal fluid. MRI of the brain showed an abnormal diffusion signal with increased apparent diffusion coefficient and little blood around the tentorium. The knowledge of the pathogenesis of PDE is still limited. The presence of Roth spots is suggestive for a pathogenic mechanism of vasogenic damage in PDE.

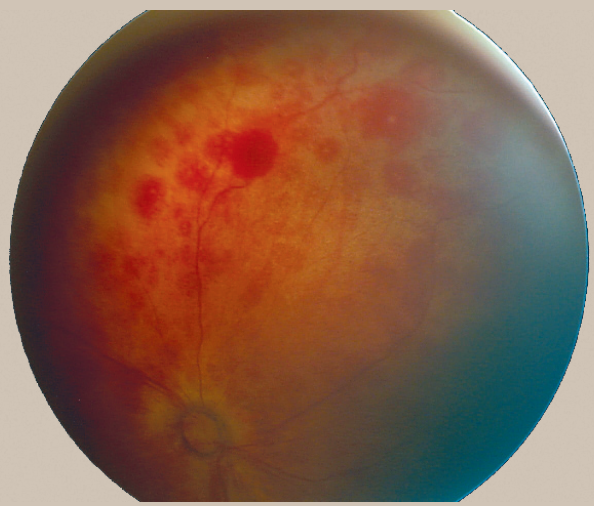
## Background

In this paper we report for the first time a patient with metabolic confirmed pyridoxine dependent epilepsy (PDE) and Roth spots. PDE is a rare metabolic defect in the degradation of lysine. This metabolic defect leads to the accumulation of $\alpha$-aminoadipic semialdehyde ($\alpha$-AASA) and indirectly to an inactivation of pyridoxal-5-phosphate, the active metabolite of pyridoxine (vitamin B6). The most striking symptom of PDE are seizures which do not respond to conventional anti-epileptic drugs but respond to pyridoxine. Knowledge of the clinical spectrum and associated abnormalities of PDE is rapidly expanding since the detection of the underlying metabolic defect and its genetic basis[2-4]. Although squint is seen in 30%[5-7] and optic nerve hypoplasia is reported in two patients[7], other true ophthalmologic abnormalities are rarely reported. Roth spots are suggestive for vasogene damage, and they might be a clue for a pathologic pathway in PDE. Therefore we believe our data could be of important additional value.

## Case presentation

A male newborn, born after an uneventful pregnancy and delivery, was admitted with generalized seizures 12 hours after birth. The convulsions were unresponsive to anticonvulsants but subsided with 100 mg pyridoxine intravenously. As can be expected in PDE, the first dose of pyridoxine led to severe respiratory depression, necessitating mechanical ventilation during a short period. The boy was clinically diagnosed with PDE on the second day of life. This diagnosis was confirmed by demonstration of highly elevated $\alpha$-AASA levels in the urine [37 mmol/mol creatinine; N < 2 mmol/mol creatinine] and a homozygous 1195 G > C antiquitin mutation. Ophthalmoscopy on the 5$^{th}$ day of life showed multiple white centred retinal haemorrhages, so called Roth spots, in both retinas (Fig. I). On follow-up these Roth spots gradually disappeared within a few months. No biochemical nor microbiological signs of infection were present in blood and liquor, bacterial cultures and viral antigens (toxoplasmosis, rubella, CMV, herpes and HIV) remained negative. Brain MRI showed some blood on the tentorium and on the falx cerebri and an abnormal diffuse signal with increased Apparent Diffusion Coefficient.

**Figure I.** Fundusphoto of the nasal side of the right eye, in the peripheral retina is multiple Roth spots (white centered retinal dot haemorrhages)



## Discussion

The observed Roth spots are rare ophthalmologic abnormalities, different from more common neonatal intra-retinal haemorrhages which are flame shaped and/or dot blot haemorrhages often related to the delivery[8]. These common postnatal retinal haemorrhages resolve usually within days[9]. Retinal haemorrhages occasionally described in metabolic diseases[10-17], are not reported as Roth spots.

Roth spots are non-specific hemorrhagic signs that occur following a variety of conditions. These conditions have in common an acute disturbances of the homeostasis contributing to retinal capillary damage and the ensuing reparative process[18,19]. Usually and most often, Roth spots are seen in patients with sub-acute bacterial endocarditis. They may also occur in several other conditions such as other infectious diseases and perinatal hypoxic encephalopathy. These were excluded in this patient[18,20].

Whether the observed Roth spots are coincidentally found in this patient with PDE, or are directly related to PDE is not clear. We can only speculate whether there is an association of Roth spots with PDE. Roth spots or retinal haemorrhages have never been reported in patients with PDE (with PLP inactivation and $\alpha$-AASA accumulation) or in other patients with pyridoxine responsiveness, e.g. hyperprolinemia type II or hypofosfataemia. A direct relation of the Roth spots with the seizures or the anti-epileptic drugs, including pyridoxine, also remains speculative.



## Learning Points

Ophthalmoscopy should be done in PDE patients.

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# Chapter IV

## General discussion and future perspectives

## General discussion and future perspectives

Pyridoxine Dependent Epilepsy (PDE) (MIM#266100) is an autosomal recessive disorder of lysine metabolism, caused by mutations in the *ALDH7A1* (antiquitin) gene on chromosome 5q31. Classically, PDE presents as a severe neonatal epileptic encephalopathy, with seizures that appear resistant to anti-epileptic drugs until the moment that pyridoxine is administered. In the general discussion of this thesis epidemiological, pathophysiological (biochemical, molecular genetic), diagnostic and outcome of treatment aspects will be discussed.

### Epidemiology of PDE in the Netherlands

PDE is a very rare disease. Epidemiological data on PDE are limited and regional differences in PDE are reported[1,2]. In 2005 we calculated a birth incidence of 1:390 000 for clinical defined probable and definite PDE in the Netherlands[3]. During the course of our study it became clear that the true incidence of PDE in the Netherlands may be (much) higher than previously thought, namely 1:200 000 born children.

The apparent increase of incidence, suggests that in the past PDE may have been under-diagnosed. This is supported by the fact that no patients born before 1991 are reported (national survey of all adult neurological departments in the Netherlands in 2006, unpublished data).

The apparent increase in the incidence of PDE in the Netherlands may be related to several factors. Nowadays PDE is confirmed by a metabolic test and if this test is abnormal it is followed by DNA examination to prove the diagnosis at the genetic level. The laboratory confirmation opens the possibility to collect data in a database and probably get a better impression of the incidence and the full spectrum of this rare disorder. There is also an increased awareness, which is reflected by the implementation of a national protocol on neonatal seizures in the Netherlands. This protocol recommends to transfer a neonate with persistent seizures to a neonatal intensive care unit, and describes the diagnostic and treatment procedures (www.nvk.nl).

Although PDE is a very rare disease, the estimated total number worldwide is significant. If the birth incidence of 1:200 000 of PDE in the Netherlands would be applied on a world wide population of 7 billion people and a world birth rate in 2011 of 19 per 1000 (wikipedia), the birth incidence would be about 600 patients per year world wide.

### Pathophysiology

Vitamin B6 is known since 1934, when György described a B factor that cured rat dermatitis. Vitamin B6 is a mixture of metabolites with several biological forms: pyridoxine, an alcohol (pyridoxol), an aldehyde (pyridoxal) and a form with an amino group (pyridoxamine), and their 5'-phosphate esters. Pyridoxine, pyridoxal and pyridoxamine-phosphate are oxidized to pyridoxal-5-phosphate (PLP) by pyridox(am)ine 5-phosphate oxidase (PNPO). PLP is the biological active form of vitamin B6. All these metabolites are generally described as if it were one metabolite, 'pyridoxine'. PLP functions as a cofactor in at least a hundred biochemical reactions.

In various animal experiments it has been shown that removal of pyridoxine from the diet results in a set of signs including dermatitis, anaemia and neurologic manifestations. Severe convulsions occur in chickens, swine's, calves and ducks. Deficient mice also can have an ataxic gait, and ducks can have difficulty in standing and paralysis of the legs. In monkeys extensive arterial lesions were found, which closely resemble human arteriosclerosis[4]. Deficient rats had opacity of the cornea[5]. Around the year 1950 a vitamin B6 deficient milk formula came to the market due to an error during the production process. Several infants that were fed with this formula developed pyridoxine deficient-like symptoms i.e. initially hyperirritability and apparent gastrointestinal colic, with regurgitation, followed by tonic posturing and generalized seizures. Hyperreactivity to sound, movement and feeding was also noted, which could precede seizures[6,7].

In 1954 Hunt suggested that PDE could best be 'described as an unusual metabolic aberration of the central nervous system in which there is a continuing high requirement of pyridoxine in excess of the normal dietary intake in order to maintain this infant in a seizure free state'[8]. In 1960 Scriver ruled out that renal loss alone could explain the findings in PDE and suggested an increased B6 requirement of the brain[9].

Previously it was suggested that the pathophysiology of PDE was related to glutamic acid decarboxylase which requires PLP to synthesize (inhibitory) gamma-amino-butyric acid (GABA) from (excitatory) glutamate[10]. However abnormalities of this enzyme were ruled out in 2000[11]. Others suggested a transport disorder of cellular B6 within the central nervous system but this hypothesis could not be proven in humans.

In 2000 Cormier-Daire et al. found linkage to chromosome 5q31, but a disease-causing gene was not identified[12]. Plecko et al reported elevations of pipecolic acid in plasma and CSF, which persisted after treatment, but the explanation for this finding was unclear at that time[13]. Finally in 2006 Mills et al demonstrated that PDE was caused by mutations in the *ALDH7A1* or the so called antiquitin gene indeed linked to chromosome 5q31[14]. The definition of the underlying defect as an inborn error of cerebral lysine degradation explained the different previous findings of pipecolic acid elevations, pyridoxine dependency, and seizures.

Although the genetic and metabolic causes of PDE have been unravelled, the 'PDE conundrum' is only partly unravelled[6] as the pathophysiology of PDE is not totally clear. In theory the pathophysiology in PDE is determined by different mechanisms like pyridoxine deficiency, accumulation of lysine metabolites ('intoxication type inborn error of metabolism'), and as the consequence of intrauterine or neonatal seizures.

Pyridoxine is known as a cofactor in the conversion of glutamate (excitatory) into GABA[10,15,16]. Pyridoxine deficiency may in some cases lead to GABA deficiency which is thought to be an important explanation for the occurrence of neonatal seizures in PDE patients. Many other enzymatic reactions need PLP as a co-factor, and toxicity or deficiency of a variety of other substrates might thus contribute to the pathogenetic mechanisms underlying the seizure disorder in PDE. The most imaginative example might be the glycine cleavage system that relies on PLP for glycine degradation. Pyridoxine is also known as a

cofactor in the sphingolipid synthesis[17,18]. Therefore pyridoxine deficiency might influence sphingolipid formation which may be responsible for white matter abnormalities seen on MRI in PDE patients[19]. Finally pyridoxine deficiency can result in extensive arterial lesions as has been observed in animals[5]. This might account for the cerebral haemorrhages seen in several patients[19] and the Roth spots we observed in one patient[20].

Accumulation of the potentially toxic lysine metabolites α-AASA, $\Delta^1$-piperideine 6-carboxylic acid (P6C), and pipecolic acid is another possible pathophysiologic mechanism in PDE. This accumulation of lysine metabolites is measurable in all body fluids[21,22] including α-AASA in amniotic fluid (unpublished data). Accumulation in the central nervous system may lead to neural cell damage and this might explain that about a third of patients are not seizure free with pyridoxine mono-therapy. Accumulation may also explain the observation of (sometimes progressive) MRI abnormalities in some PDE patients, like ventriculomegaly and white matter abnormalities[19], even in antenatal treated patients[23]. Importantly, pyridoxine supplementation decrease the amounts of accumulated lysine metabolites[13]. The impact of dietary lysine restriction, aiming to further decrease accumulation of lysine metabolites has to be studied. The first observational study with lysine restriction is promising and will be discussed below[24].

Like some other metabolic diseases PDE can result in cerebral malformative consequences. For instance, brain malformations can be observed in Congenital Disorders of Glycosylation[25], Smith-Lemli-Opitz syndrome[26], peroxisomal disorders[27] and others. The observed brain development abnormalities in PDE are Corpus Callosum Dysgenesia, Mega Cisterna Magnum and focal lesions as in the globus pallidus, peduncle cerebellum, sub-cortex and/or skull-bone[19].

In conclusion, although PDE is nowadays known to be caused by a disturbance of the lysine catabolism in the cerebral nervous system our knowledge of the pathophysiology of PDE is still limited. Further research is necessary to increase our insights in the underlying disease mechanisms. Better understanding is required to improve treatment strategies and long-term outcome of patients with PDE.

**Diagnosis of PDE**

After the first publication by Hunt in 1954, neonatologists and paediatric neurologists kept struggling to identify PDE in patients with therapy resistant seizures[8]. The development of clinical criteria was very useful, and supplied the clinician with some help in the differential diagnostic work-up and choices with regard to treatment[28]. Baxter defined definite PDE as neonates, infants, or young children with recurrent (two or more) seizures of any type that ceased within seven days after the administration of oral pyridoxine (usual dose, 30 mg/kg/day, minimum dose, 15 mg/kg/day) or within 30 minutes of intravenous pyridoxine (usual dose 100 mg, minimum dose, 50 mg), and recurred when pyridoxine supplementation was withdrawn, re-introduction of pyridoxine should finally cease the seizures again. Possible cases were defined as above, but without attempt to withdraw and re-introduce pyridoxine. Recurrence of seizures while receiving pyridoxine treatment was an exclusion criterion, unless the recurrence occurred during a febrile illness. Two subgroups were defined as probable PDE because no formal trial of withdrawal was undertaken. In one subgroup,

seizures stopped after a single dose of pyridoxine, recurred later, and again responded to pyridoxine, which was then continued. A second subgroup comprised of children with an affected sibling whose seizures began at the same age and responded to pyridoxine.
Although clinical criteria were often helpful, identification of PDE remained difficult in about a third of patients[10,29].

**Pitfalls in clinical diagnosis**

Pitfalls in the diagnostic process of PDE are common. Several studies are presented in this thesis[21,30] that show the problems to identify or to exclude PDE in patients with therapy resistant seizures (TRS), when only clinical criteria are available. One of these pitfalls is that some neonates with TRS due to PDE present with problems that are very common in neonatal intensive care units like hypoxic ischemic encephalopathy, hypoglycaemia or neonatal cerebral haemorrhage. In those cases, these conditions may be considered the full explanation of the TRS while they are in fact only secondary to the underlying disorder PDE[30]. Some children with PDE present with feeding problems while seizures develop only later[10]. Some children with PDE have specific EEG features while in others the EEG is normal prior to the first gift of pyridoxine, despite clinical seizure activity[3]. In most patients with PDE, seizures start in the first week of life, but in some children seizures only start several months after birth. Although most patients do not respond to anti-epileptic drugs at all, some have a good initial clinical response on first line anti-epileptic drugs such as phenobarbitone, midazolam and/or phenytoin[10,31,32]. Most, but not all patients respond to the first gift(s) of pyridoxine with a dramatic decrease of seizure frequency. Not only patients with PDE, but also infants with other epileptic encephalopathies may benefit from pyridoxine supplementation[30,33,34]. Paradoxically, however an increase of seizure activity following pyridoxine therapy has also been reported in children with epilepsy due to other causes than PDE[35,36].

Some children need a large first dose of pyridoxine, up till 500 mg, before a response occurs and seizures disappear[31] (this Thesis) and in some children the EEG normalizes only 5 days after the first pyridoxine dose[30]. Notably, about 30% of children with PDE do not remain seizure-free on a maintenance dose of pyridoxine but require additional conventional anti-epileptic drugs[19,37,38]. Some children benefit from additional folic acid supplementation and some PDE patients even have been treated successfully with mono-therapy folic acid (see also discussion below)[39]. In summary, considering all mentioned pitfalls and the diverse clinical picture, the diagnosis of PDE on clinical criteria is difficult in a significant proportion of patients.

In this thesis we performed metabolic studies[21] in clinical diagnosed PDE patients[3]. In most patients with definite, possible or probable PDE, α-AASA dehydrogenase deficiency was demonstrated. Metabolic examination of urine, to determine the concentration of α-AASA, proved to be a reliable tool to confirm or to exclude PDE. The assumption is that the sensibility and specificity of elevated concentrations of urinary α-AASA are high. It is important to note that α-AASA, which was initially thought to be a specific and unique marker for PDE, has recently been found elevated in both molybdenum cofactor deficiency and isolated sulphite oxidase deficiency[40]. This finding has to be taken in account for diagnosis and treatment in patients with neonatal therapy resistant seizures. An advantage

of metabolic examination is that the potentially dangerous 'trial of withdrawal of pyridoxine', classically used to prove PDE, can now be avoided. Metabolic testing for PDE is therefore advised in all children till the age of 18 months with therapy resistant seizures.

Intra-uterine diagnosis of PDE is challenging. DNA examination of the foetus is possible after amniocentesis or chorionic villi biopsy, which are invasive procedures, and as such not without risks for the mother or the foetus. We recently investigated the urine of a pregnant woman who was carrying a foetus that was potentially affected with PDE, and who used pyridoxine during this second pregnancy. Her first child was diagnosed with (metabolic and DNA confirmed) PDE. Examination of the maternal urine sample showed no increased of α-AASA levels (not published). After birth pyridoxine treatment of the child was continued and PDE was diagnosed by demonstrating increased α-AASA concentrations in the baby's urine, and two mutations in the *ALDH7A1* gene.

In neonates with TRS, whether or not due to PDE, intravenous treatment with pyridoxine induces EEG changes. These changes were found neither sensitive nor specific for PDE[30]. This study demonstrated that the EEG response to pyridoxine should be interpreted with caution and it was concluded that, irrespective of the EEG response to pyridoxine, neonates and infants suspected from PDE should continue to receive pyridoxine until PDE is diagnosed or excluded by biochemical and/or genetic analysis. New technical developments regarding digital analysis of EEG's, in particular (automated) quantification of multichannel EEG's might provide novel tools to study PDE[41-43].

**Outcome and Treatment / Outcome of Treatment**

Now that the cause of PDE has been identified and some ideas about underlying disease mechanisms and additional possibilities for treatment are formulated (see above), future studies should be focused on long-term outcome. Long term outcome of PDE patients is not invariably good. Our patient cohort, as other cohorts, showed that most patients are intellectually disabled: roughly 75% of them having an IQ < 85. About two-third of PDE patients attend schools for special education[19,37,38]. About 30% of patients need anti-epileptic drugs besides pyridoxine. Another important issue is that too little is known about optimal treatment schedules (including optimal daily dose of pyridoxine, see below) in relation to outcome[19]. At the moment most PDE patients receive a daily dose of 15 to 30 mg/kg/day, in fact based on expert opinion or experiences in single cases or small series[29]. In 1996 the optimum daily dose of pyridoxine was studied in a small cohort (n = 6). A daily dose of less than 2 mg/kg appeared to be insufficient. A marked improvement of IQ and behaviour was seen in some patients after increase of the daily dose[1]. However we could not find a relation between pyridoxine maintenance dose and outcome[19]. Also the timing of treatment seems important. As delay in pyridoxine treatment probably worsens outcome. Finally, one Dutch young adult recently reported feelings of depression, which resolved after increase of her daily dose from 100 to 200 mg pyridoxine (unpublished data). Altogether, the current advised daily dose for pyridoxine is thus based on expert opinions with limited patients observations, without studies of the pyridoxine levels in the cerebrospinal fluid and without structured, prospective studies. Only recently normal values for cerebrospinal fluid B6 vitamers in preterms have been reported[44]. There have been no controlled trials in neonates with PDE that studied long-term outcome in relation to daily dose of pyridoxine.

These studies should be done and they should also study side effects of pyridoxine in PDE and non-PDE patients, as side effects have been reported[35,45]. Currently, it is assumed that patients with PDE should receive 15 mg/kg/day (maximum 150 mg/day) pyridoxine, and only in exceptional cases, e.g. with intractable seizures necessarily to reach and maintain seizure control, higher dosages seem warranted.

Antenatal start of treatment was associated with a favourable outcome[23]. The benefits of antenatal treatment were only studied in a few individual cases, and the results of these studies should be interpreted with caution. They provide, however, a reasonable argument to supplement mothers of PDE patients with pyridoxine during subsequent pregnancies, in order to prevent foetal and neonatal seizures, as well as birth-related complications, and improve neurodevelopmental outcome. The maternal advised daily doses should be 50 to 100 mg daily. Maximal 100 mg because higher maternal doses of pyridoxine might potentially cause neuronal hyperexcitability in non-PDE neonates[35].

It has long been thought that folinic acid responsive seizures and PDE were two separate but resembling disorders. However all patients diagnosed with folinic acid responsive seizures in the literature, have recently been shown, both biochemically and genetically, to suffer from antiquitine deficiency, i.e. PDE. This indicated that folinic acid can be of additional benefit in PDE patients, though the biochemical basis for this is unclear[39]. To date it is unclear why folic acid treatment is successful in some PDE patients.

The treatment with pyridoxine compensates the chemical inactivation of PLP, but the accumulation of lysine degradation metabolites does not normalise[24]. These accumulating metabolites i.e. $\alpha$-AASA, P6C and pipecolic acid are potentially neurotoxic compounds. The increased levels of lysine degradation metabolites might be responsible for the limited success of pyridoxine treatment, as about 75% of patients have developmental delay, 30% need additional anti-epileptic drugs and progressive cerebral MRI abnormalities are observed in patients[19]. Standard dietary treatment for inborn errors of metabolism consists of substrate reduction. For instance in glutaric aciduria type I, also an inborn error of the degradation of lysine, prognosis can be improved with a diet low in lysine and with carnitine supplementation. So for PDE, dietary lysine reduction can reduce accumulation of lysine substrates and this might improve outcome. The first observational study on dietary lysine restriction presented hopeful results[24]. The diet was well tolerated with good compliance, no side effects were reported. Reduced levels of lysine metabolites were measured in all patients. Seizure control and improvement of age-appropriated skills was observed in most. So additional treatment with a lysine restricted diet is hopeful but before such a diet therapy becomes a mainstay of treatment, more evidence has to be generated.

Because of the rarity of PDE, international collaboration is necessary, since only international, multi-centre collaboration might result in studied cohort sizes which allow firm evidence. These studies need modern methodologies which permit firm statistical relevant conclusions. The endpoint of such studies should be focused on developmental outcome. The requirements for such studies are funding, a structured international database, and a medical ethical approved research protocol.

In 2003 Baxter reported that PDE remained a clinical and biochemical conundrum. In the last years important progress has been made to understand PDE. However, we can only state that this conundrum is partly unravelled and prognosis of children with PDE has not been improved. So we should continue our efforts and research to understand PDE and improve outcome.

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# Chapter V

## Summary

## Summary

Pyridoxine Dependent Epilepsy (PDE, MIM#266100) is a rare autosomal recessive disorder. In most patients PDE is characterized by neonatal seizures that do not respond to classic anti-epileptic drugs but do respond after supplementation of pyridoxine.

From 2006 two Dutch groups, under the leadership of Prof. Dr. Michèl Willemsen, Department of Pediatric Neurology, Radboud University Nijmegen, Medical Centre, Nijmegen and Prof. Dr. Cornelis Jakobs, Metabolic Unit, Department of Clinical Chemistry, VU University Medical Centre, Amsterdam collaborated on a PDE research project. In this thesis we studied epidemiological, biochemical, molecular genetic and clinical aspects (diagnostic and outcome) of the Dutch PDE patients (11 patients in 2005; and 22 patients in 2012).

Epidemiologic data on PDE are scarce and national data were only available from the UK and Ireland (1:783 000). In 2005, we studied the epidemiology of PDE based upon clinical criteria in the Netherlands (**Chapter I.b**). Nationwide all departments of paediatrics (n = 113; response rate 67%) and departments of paediatric or neonatal neurology (n = 17; response rate 94%) were asked to report patients with PDE. Thirteen patients were reported and we classified them as having definite (n = 4), probable (n = 3), or as possible (n = 4) PDE. Two patients did not meet clinical criteria for either of these groups. Based on these data, a birth incidence of PDE in the Netherlands was calculated to be 1:396 000 for definite and probable cases, and 1:252 000 when possible cases were also included. The study showed that the diagnosis in the past was often made without performance of a formal 'trial of withdrawal'. We concluded that regional differences in diagnostic skills were unlikely to account for the different incidences. It was postulated that a founder effect in the Netherlands might explain the difference in birth incidence between the United Kingdom and the Netherlands. Furthermore, it seemed quite reasonable to us to assume that some patients with PDE may never have been recognised, i.e. diagnosed, and (thus) may have missed the chance to be treated appropriately. At that moment we concluded that a better knowledge of the disease entity and the clinical criteria needed for establishment of the diagnosis, as well as better understanding of the underlying disease mechanism, would contribute to increased awareness, earlier diagnosis, and more adequate management of PDE.

After completion of this epidemiological study the metabolic mechanism of PDE was elucidated. In 2006, α-aminoadipic semialdehyde (α-AASA) dehydrogenase deficiency was identified as the cause of PDE (Mills 2006). In 2007, **Chapter II.a**, we re-evaluated the series of clinically diagnosed Dutch PDE patients described in Chapter I.b, by measuring α-AASA and PA levels in urine and plasma. In all patients with clinically definite PDE, and in most patients with probable or possible PDE, the clinical diagnosis of PDE could be confirmed at the metabolite level. This study confirmed that non-invasive urinary screening for α-AASA provides a reliable tool to diagnose PDE.

In **Chapter II.b** the reference values for urinary P6C and α-AASA were determined. We studied 91 urine samples from neonates admitted to a level IIIa NICU in Veldhoven,

the Netherlands. Additional we studied 100 urine samples in older children, to establish reference values for α-AASA and P6C for all age groups. Urinary concentrations of P6C and α-AASA values correlated positively with protein intake, and negatively with gestational age and body weight, no correlation with gender or any studied neonatal IC condition or treatment was found.

After 2006 the assessment of urinary α-AASA is **the** diagnostic laboratory test for PDE. α–AASA is in spontaneous equilibrium with its cyclic form $\Delta^1$-piperideine-6-carboxylate (P6C). Ongoing diagnostic screening and monitoring revealed that in some individuals, with apparently milder *ALDH7A1* variants, and patients co-treated with a lysine restricted diet, urinary α-AASA was only modestly increased. This prompted us to investigate the diagnostic power and added value of the assessment of urinary P6C, as P6C measurement is easier to perform, compared to α-AASA (**Chapter II.b**). In all 40 urine samples from 35 individuals with proven PDE, we detected increased levels of P6C. Therefore, we concluded that the diagnostic strength of the assessment of urinary P6C and α-AASA is comparable.

Following the first identification of pathogenic mutations in the α-AASA dehydrogenase (*ALDH7A1*, Antiquitin) gene in 2006, we analyzed the DNA of the Dutch biochemically proven PDE patients (**Chapter II.c**). We identified three different mutations in the Dutch patients: the common c.1195G > C, p.Glu399Gln mutation; a novel missense mutation (c.1348T > A; p.Cys450Ser) and an intriguing "silent" mutation (c.750G > A; p.Val250GlyfsX23) in *ALDH7A1*. We concluded that the high incidence of the c.1195G > C mutation is a strong argument for a founder effect in the Netherlands - as we had postulated in 2005, Chapter I.b. This study also illustrates the importance of mRNA studies when a seemingly non-pathogenic or 'silent' variant is detected, especially when increased urinary levels of α-AASA are measured without the identification of causative mutations in the *ALDH7A1* gene.

Because of the rarity of PDE clinical cohort studies in PDE are uncommon, as they take great effort to collect data. Only 200 patients have been described in literature, in about 130 reports (Stockler 2012) and most reports describe less than 5 patients, which limits conclusions.

Prompt recognition and treatment of PDE is probably important for prognosis. We studied immediate EEG alterations in neonates following intravenous pyridoxine, **Chapter III.a**. Our hypothesis was that in neonatal Therapy Resistant Seizures (TRS), direct EEG alterations would discriminate PDE from non-PDE patients. In ten neonates with TRS (6 PDE, 4 non-PDE), we compared on-line EEG alterations by pyridoxine-IV. EEG segments were visually and digitally analyzed for average background amplitude and total- and relative- power. In 3 of 10 (2/6 PDE and 1/4 non-PDE neonates), pyridoxine-IV caused flattening of the EEG amplitude and attenuation of epileptic activity. Quantitative EEG alterations by pyridoxine-IV consisted of EEG responses ($p < 0.05$), similar in PDE and non-PDE for central amplitude, total power and relative power. In conclusion, our hypothesis had to be rejected, as in neonatal TRS, pyridoxine-IV induces non-specific EEG responses that neither identify nor exclude PDE. Pyridoxine supplementation should be continued in neonates with TRS until PDE is diagnosed or excluded by metabolic and/or DNA analysis.

In **Chapter III.b** we studied the effects of antenatal treatment in PDE as incidental reports suggested that antenatal treatment of PDE may improve neurodevelopmental outcome. Two families with PDE are reported. Antenatal treatment was instituted during the second pregnancy in each family (50 and 60 mg pyridoxine daily from 3 and 10 weeks of gestation, respectively). Perinatal characteristics and neurodevelopmental outcome were compared between the untreated and treated child within each family. Meconium stained amniotic fluid was present in both first pregnancies and abnormal foetal movements were noticed in one. In the treated infants, pregnancy and birth were uncomplicated and neonatal seizures were prevented. In family A, DQ was 73 and 98 in the antenatally untreated and treated child respectively. In family B, IQ's with and without fetal therapy were 80 and 106 respectively.

The results presented in this study suggest that antenatal pyridoxine supplementation in selected families may be effective in preventing intrauterine seizures, decreasing the risk of complicated birth, and improving neurodevelopmental outcome in PDE.

The aim of the study in **Chapter III.c** was to determine long-term developmental outcome and relating factors between patient characteristics and follow-up data. Fourteen patients were included (4 males, 10 females; 11 families; median age at assessment 6 years; range 2.5 – 16 years).Pyridoxine was started antenatally in two children, in the first week of life in five, in the first month of life in three, or after the first month of life (range 2.5 – 8 months) in four. No children were physically disabled; however, only five walked at 2 years of age. Mental development was delayed in most: median IQ or developmental index was 72 (SD 19). Pyridoxine controlled seizures in 10 of 14 children, four needed additional antiepileptic drugs. Seizure persistence, antiepileptic drugs (other than pyridoxine), EEG background, and epileptiform activity were not associated with outcome. On neonatal MRI, structural and white matter abnormalities occurred in five of eight children; on follow-up, the number of abnormal MRI findings had increased. Delayed initiation of pyridoxine medication and corpus callosum abnormalities were significantly associated with unfavourable neurodevelopmental outcome, but normal follow-up imaging did not predict a good outcome. These results make clear that the outcome for patients with PDE is still not favourable and that individual outcome cannot be predicted by the evaluated characteristics.

In **Chapter III.d** we reported one PDE patient. On ophthalmoscopy on the 5$^{th}$ day bilateral multiple white centred retinal haemorrhages, so called Roth spots, were observed. These Roth spots disappeared after a few months. MRI of the brain showed an abnormal diffusion signal with increased Apparent Diffusion Coefficient and little blood around the tentorium. Roth spots are non-specific hemorrhagic signs that can occur in a variety of conditions of acute systemic insults in homeostasis, most often infections, which relate to retinal capillary damage and the ensuing reparative process – none were present in this patient. Further research is necessarily to determine whether Roth spots are a regular finding and related to PDE.

# Addendum

## Nederlandse samenvatting
## Dankwoord
## Curriculum vitae

## Samenvatting

Pyridoxine Afhankelijke Epilepsie, Pyridoxine Dependent Epilepsy (PDE, MIM#266100) is een zeldzame autosomaal recessieve aandoening. Bij de meeste patiënten wordt PDE gekenmerkt door convulsies (stuipen) bij pasgeborenen. Deze reageren niet op de anticonvulsieve medicijnen die gebruikt worden bij pasgeborenen maar verdwijnen wel na toediening van pyridoxine (= vitamine B6).

Vanaf 2006 werken in Nederland twee groepen samen in hun wetenschappelijk onderzoek rond PDE. Eén onder leiding van Prof. Dr. Michèl Willemsen, Afdeling Kinderneurologie, Radboud Universiteit Nijmegen, Medisch Centrum, Nijmegen en de ander onder leiding van Prof. Dr. Cornelis Jakobs, Metabole Unit, Afdeling Klinische Chemie, VU Universitair Medisch Centrum, Amsterdam. In dit proefschrift werden de epidemiologische, biochemische, moleculair genetische and klinische aspecten (Diagnose en Prognose) van de Nederlandse PDE patiënten (11 patiënten in 2005; 22 patiënten in 2012) beschreven.

Epidemiologische data met betrekking to PDE zijn schaars gepubliceerd. Nationale data zijn alleen vanuit Engeland en Ierland (1:783 000) gemeld. In 2005, bestudeerden wij de epidemiologie van PDE, gebaseerd op klinische kenmerken in Nederland (**Chapter I.b**). Schriftelijk werden alle kinderafdelingen (n = 113; response 67%) en alle kinderneurologische afdelingen (n = 17; response 94%) gevraagd hun PDE patiënten te melden. Dertien patiënten werden gemeld en zij werden door ons geclassificeerd als 'definite' (n = 4), 'probable' (n = 3), of 'possible' (n = 4) PDE patiënt. Twee patiënten voldeden aan geen van de gestelde klinische criteria. Op basis van deze data werd een geboorte incidentie voor Nederland berekend van 1 op 396 000 voor 'definite' en 'probable' PDE, en van 1 op 252 000 als ook de kinderen met 'possible' PDE worden geïncludeerd. Deze studie liet zien dat de diagnose PDE in het verleden vaak was gesteld zonder een formele 'trial of withdrawal'. Tevens werd geconcludeerd dat landelijke verschillen in diagnostische skills waarschijnlijk niet de verklaring zijn voor de gevonden verschillen in incidentie tussen de verschillende landen. Het verschil in geboorte incidentie tussen Nederland en Engeland is mogelijk te verklaren door een 'founder' effect in Nederland. Het leek ons verder aannemelijk dat sommige patiënten nooit worden herkend en dus nooit worden gediagnosticeerd met PDE, en derhalve niet de kans hebben om een adequate medische behandeling te ontvangen. De conclusie van de studie was dat een betere kennis van de klinische criteria van PDE, alsook een beter begrip van de onderliggende ziekte mechanismen, zou kunnen leiden tot een hogere alertheid voor PDE, een vroegere diagnose en een adequate behandeling.

Na publicatie van deze epidemiologische studie werd het metabole defect van PDE gevonden. Een tekort van het enzym $\alpha$-aminoadipine semialdehyde ($\alpha$-AASA) dehydrogenase in het lysine metabolisme, bleek verantwoordelijk voor PDE (Mills 2006). Daarna, **Chapter II.a**, herevalueerden wij de patiënten met klinisch gediagnosticeerd PDE, Chapter I.b, door bij deze patiënten het $\alpha$-AASA en het pipecoline zuur in urine en plasma te meten. Bij alle patiënten met 'definite' PDE en bij de meeste patiënten met 'probable' of 'possible' PDE, kon de klinische diagnose van PDE worden bevestigd door middel van metabool onderzoek. Deze studie bevestigde dat non-invasief urine onderzoek van $\alpha$-AASA, een betrouwbare methode is om PDE te diagnostiseren.

In **Chapter II.b** worden de referentie waarden voor P6C en α-AASA in de urine beschreven. Hiervoor werden 91 urine monsters van pasgeborenen die waren opgenomen op de NICU in Veldhoven onderzocht. Om de leeftijd specifieke referentie waarden voor α-AASA and P6C te bepalen werden 100 urine monsters onderzocht van oudere kinderen. De urine waarden voor P6C en α-AASA zijn positief gecorreleerd met eiwit intake en negatief met zwangerschapsduur en lichaamsgewicht. Geen correlatie werd gevonden met geslacht of neonatale Intensive Care aandoening.

Sinds 2006 is de bepaling van α-AASA in urine, **de** diagnostische laboratorium test voor PDE. α-AASA blijkt in spontaan evenwicht te zijn met zijn cyclische vorm $\Delta^1$-piperideine-6-carboxylate (P6C). Inmiddels blijkt dat sommige kinderen, mogelijk kinderen met een mildere *ALDH7A1* variant, en kinderen met een lysine beperkt dieet, dat in de urine soms slechts licht verhoogde α-AASA waarden worden gevonden. Daarom werd besloten te onderzoeken wat de toegevoegde waarde is van de urine bepaling van P6C, omdat deze bepaling gemakkelijker is te verrichten is dan de meting van α-AASA. In 40 urine monsters van 35 PDE patiënten, werden vergelijkbare verhoogde waarden van P6C gemeten. Er werd daarom geconcludeerd dat de diagnostische waarde van P6C en α-AASA in urine vergelijkbaar is.

Na de identificatie van de pathogene mutaties in het α-AASA dehydrogenase (*ALDH7A1*, Antiquitin) gene in 2006, werd het DNA van de Nederlandse patiënten met biochemisch bewezen PDE (**Chapter II.c**) geanalyseerd. Bij de Nederlandse patiënten werden drie verschillende mutaties gevonden: de in Nederland frequente c.1195G > C, p.Glu399Gln mutatie; een nieuwe 'missense' mutatie (c.1348T > A; p.Cys450Ser) en een "silent" mutatie (c.750G > A; p.Val250GlyfsX23) in het *ALDH7A1* gen. Er werd geconcludeerd dat de hoge incidentie van de c.1195G > C mutatie een sterke aanwijzing is voor een 'founder' effect in Nederland – zoals reeds eerder was gepostuleerd in 2005, Chapter I.b. Deze studie illustreert ook het belang van mRNA studies bij de vondst van ogenschijnlijke niet-pathogene of 'silent' varianten, met name als verhoogde urine waarden worden gemeten van α-AASA, zonder de identificatie van verklarende pathogene mutaties in het *ALDH7A1* gen.

Klinische cohort studies van PDE patiënten zijn nauwelijks gepubliceerd, omdat PDE zeer zeldzaam is en omdat het een grote inspanning vergt om voldoende data te verzamelen. Slechts 200 patiënten zijn beschreven in de literatuur in ongeveer 130 studies (Stockler 2012) en de meeste studies beschrijven minder dan 5 patiënten, hetgeen de conclusies per studie beperkt.

Directe herkenning en behandeling van PDE lijkt belangrijk voor de prognose. De EEG veranderingen bij pasgeborenen direct na intraveneuze (IV) toediening van pyridoxine werd bestudeerd, **Chapter III.a**. De hypothese was dat bij kinderen met neonatale Therapie Resistente Stuipen (TRS), directe EEG veranderingen het onderscheid mogelijk zou maken tussen PDE en niet-PDE patiënten. Bij tien pasgeborenen met TRS (6 PDE, 4 niet-PDE), werden de directe online EEG veranderingen na pyridoxine–IV vergeleken. EEG segmenten werden visueel en digitaal geanalyseerd, gericht op gemiddelde achtergrond, en totale- en relatieve- power. Bij 3 van de 10 pasgeborenen (2 van de 6 PDE plus 1

van de 4 niet-PDE pasgeborenen), veroorzaakte pyridoxine-IV afvlakking van de EEG amplitude en verdwijnen van de epileptische activiteit. Kwantitatieve EEG veranderingen voor zowel centrale amplitude, totale power en relatieve power, direct na pyridoxine-IV veroorzaakte een EEG respons ($p < 0,05$), die vergelijkbaar was tussen PDE en niet–PDE pasgeborenen. Uit dit onderzoek werd geconcludeerd dat het niet mogelijk is PDE of niet-PDE te onderscheiden op basis van de EEG respons na pyridoxine. Derhalve moet pyridoxine toediening worden gecontinueerd bij pasgeborenen met TRS totdat PDE is eëxcludeerd door middel van metabool en of DNA onderzoek.

In **Chapter III.b** werden de effecten van antenatale behandeling bij PDE bestudeerd. Sommige studies suggereerden namelijk dat antenatale behandeling de psychomotore ontwikkeling van PDE patiënten zou kunnen bevorderen. In 2 families met eerder een kind met PDE kreeg de moeder tijdens deze tweede zwangerschap, respectievelijk dagelijks 50 en 60 mg pyridoxine vanaf de $3^e$ respectievelijk de $10^e$ zwangerschap week. Na de geboorte werd bij deze tweede kinderen PDE geconstateerd middels een verhoogd $\alpha$-AASA. Binnen één gezin werden de perinatale karakteristieken en de psychomotore uitkomst vergeleken tussen het kind dat wel en het kind dat niet antenataal behandeld was. Meconium houdend vruchtwater was aanwezig bij beide eerste zwangerschappen en abnormale kindsbewegingen werden opgemerkt door één moeder. Bij de behandelde kinderen was de zwangerschap en geboorte ongecompliceerd en neonatale stuipen werden voorkomen. Bij familie A was het IQ respectievelijk 73 en 98 bij het antenataal niet en het antenataal wel behandelde kind. In familie B was het IQ respectievelijk 80 en 106.

De resultaten van deze studie suggereren dat antenatale pyridoxine behandeling in geselecteerd families effectief zou kunnen zijn om intra-uteriene stuipen te voorkomen, het risico op een geboorte complicatie te verminderen en de psychomotore ontwikkeling van het kind te verbeteren.

Het doel van de studie in **Chapter III.c** was om te bepalen wat de psychomotore ontwikkeling op de lange termijn is en welke factoren hierbij van invloed zijn. Veertien patiënten deden mee aan het onderzoek (4 jongens en 10 meisjes; 11 families; mediane onderzoeksleeftijd was 6 jaar; spreiding van 2,5 tot 16 jaar). Pyridoxine was antenataal gestart bij twee kinderen (Chapter II.b), in de eerste levensweek bij vijf, in de eerste levensmaand bij drie of gestart na de eerste levensmaand bij vier kinderen (spreiding 2,5 tot 8 maanden). Geen van de kinderen was lichamelijke gehandicapt; maar slechts vijf kinderen konden los lopen op de leeftijd van 2 jaar. De mentale ontwikkeling was vertraagd bij de meeste kinderen: het mediane IQ of ontwikkeling index was 72 (SD 19). Pyridoxine controleerde de convulsies bij 10 van de 14 children, vier kinderen hadden additionele anti-epileptische medicijnen nodig. Blijvende convulsies, het gebruik van anti-epileptische medicijnen (anders dan pyridoxine), EEG achtergrond patroon en EEG epileptiforme activiteit waren niet geassocieerd met de psychomotore ontwikkeling. Op de neonatale MRI werden structurele afwijkingen en witte stof afwijkingen gezien bij vijf van de acht kinderen; bij follow-up was het aantal abnormale MRI bevindingen toegenomen. Late pyridoxine behandeling en Corpus Callosum afwijkingen waren significant geassocieerd met een ongunstige psychomotore ontwikkeling, maar een normale MRI bij follow-up voorspelde geen ongestoorde psychomotore ontwikkeling. Deze resultaten maken duidelijk

dat de prognose van kinderen met PDE nog steeds niet goed is en dat de individuele ontwikkeling niet voorspeld kan worden.

In **Chapter III.d** wordt één PDE patiënt beschreven met bilateraal multiple retina bloedingen met een wit centrum gezien, zogenaamde Roth spots. Geleidelijk verdwenen deze Roth spots na een paar maanden. MRI van de hersenen toonde een abnormaal diffusie signaal met verhoogd Apparent Diffusion Coefficient en wat bloed rond het tentorium. Roth spots zijn niet-specifieke bloedinkjes welke bij verschillende aandoeningen kunnen ontstaan waarbij sprake is van een plotse systemische verandering in de homeostase, meestal betreft dit infecties, die gerelateerd zijn met beschadigingen van het retinale capillaire vaatbed en het aansluitende lokale genezingsproces in de retina. Geen van de bekende geassocieerde aandoening was aanwezig bij deze PDE patiënt. Mogelijk zijn Roth spots geassocieerd met PDE, verdere research is nodig om te bepalen of Roth spots regelmatig gevonden worden bij PDE en wat de relatie is met PDE.

## Dankwoord

De weg van de wetenschap is als het leven. Ga elke dag op stap met een doel, vastberaden, met geduld en passie. Gestaag zul je je doel bereiken. Op de reis van dit onderzoek heb ik mij nooit gehaast en vooral genoten van alle plekken waar ik geweest ben. En ik verheug mij op de plekken waar ik ooit nog hoop te komen. Samen kom je ergens, alleen bereik je niks. Een ieder die ik op mijn reis ben tegen gekomen en die mij geholpen heeft wil ik bij deze bedanken, mij realiserende dat mijn reis in Pyridoxine Afhankelijke Epilepsie nog niet afgelopen is. Er zijn te veel mensen die ik moet en wil bedanken en helaas zal ik ook velen vergeten, waarvoor mijn excuses. Enkelen wil ik toch met nadruk bedanken.

Beste Michèl, Prof. Dr. M.A. Willemsen. In 2006 kon ik na jouw telefoontje niet bevroeden waar ik zou komen, alhoewel jij meteen benadrukte dat op dit onderwerp een promotie mogelijk zou zijn. Ik dank je voor je integere steun en uitstekende hulp op de momenten dat ik die nodig had. Jij begrijpt waar mijn talenten liggen en wat mijn verbeterpunten zijn. Mijn dank. Ook ging jij op de juiste momenten in de wind staan om één en ander te regelen, zodat ik in de luwte verder kon werken aan de inhoud van dit boekje. Gaarne heb ik gewacht zodat jij mijn promotor zou kunnen zijn. Ik hoop dat we in de komende jaren verder kunnen blijven samenwerken aan de pyridoxine problematiek.

Prof dr C Jakobs, beste Karel. Dank dat ik, als buitenstaander in jullie topkeuken van de metabole ziekten heb mogen werken. Ik was altijd weer verbaasd hoe groot jullie inzicht is in de biochemische mechanismen van dit ziektebeeld. Het is voor mij een eer dat ik als één der laatste bij jou mag promoveren.

Dr. E Struijs, beste Eduard. Regelmatig hebben we gesproken over AASA en pipecolinezuur, maar bovenal praten we graag over snoeken. De uitspraak 'Je moet vissen anders kun je niet vangen' gaat niet over vissen maar over onze gemeenschappelijke attitude in het leven, blijf bezig en je zult inzichten opdoen en verbeteringen vinden.

Dr J. Been, beste Jasper, dankzij jou heb ik mijn plekje gekregen op de weg van onderzoek. Samen hadden we een idee. Ik bepaalde een koers en jij zorgde er voor dat we op de weg bleven. Samen kwamen we aan bij ons reisdoel. Dank dat ik met jou heb mogen samen werken.

Dr. D Sival, beste Deborah, groot is mijn dank hoe goed jij kunt 'kleien'. Ik heb dat talent niet, laat staan het geduld. Zonder jouw hulp was het hoofdstuk over het neonatale EEG nooit geworden wat het nu is.

Lucas Dijck, dank voor je hulp bij het bestuderen van de EEG's. Maar bovenal dank voor gesprekken en de kop koffie die ik met jou mocht drinken.

Beste Feico en Whalter. Beste collega's. Dank voor jullie hulp in de voorbereiding respectievelijk de afronding van meerdere manuscripten. Dank ook hoe jullie mijn paranimfale-dilemma hebben opgelost. Ik hoop nog jaren intensief met jullie te mogen samenwerken in Veldhoven.

Beste Saskia Houterman, mijn dank is groot hoe jij met flair, mij door de lastige epidemiologie hebt geleid.

Alle kinderneurologen en alle neonatologen in Nederland, klinische chemie MMC, collega kinderartsen, secretariaat dank voor alle data die ik met jullie directe of indirecte hulp mocht verzamelen en voor jullie geduld dat ik jaren lang op dinsdag ochtend probeerde een stap voorwaarts te maken in dit onderzoek. Beste Francien, bovenstaande is natuurlijk bovenal voor jou van toepassing.

Beste Ouders en kinderen met pyridoxine afhankelijke epilepsie, dank voor al jullie niet aflatende medewerking en dank voor het vertrouwen dat ik van jullie heb mogen ontvangen.

Mijn ouders wil ik danken voor alles kansen die zij voor ons kinderen hebben gecreëerd. Ik dank mijn broer en zussen voor de lol in de familie en de kracht die we gezamenlijk kunnen ontwikkelen.
Ine, Anita, Suzanne, Pa, schoonouders. Graag had ik jullie op deze dag aanwezig gezien. Dagelijks zijn onze gedachten bij jullie.

Lieve Marita, Rosalie, Benjamin, Gidius, wat zou ik zijn zonder jullie. Marita, mijn dank is groot voor je liefde voor mij. Al ben ik een gewone man, 'behind me, there is a great woman'. Rosalie, Benjamin, Gidius, niks is teveel om voor jullie te doen. Van harte hoop ik dat jullie je talenten ten volle kunnen ontwikkelen. Ik hoop dat we nog 1000 zondag-ochtend-ontbijtjes mogen hebben.

## Curriculum Vitae

Levinus Adrianus (roepnaam Vinus) Bok, werd op 26 april 1958 geboren in de Noord Oostelijke Polder. Zijn ouders, Wijnand Bok en Truus Bok - van Eeten zijn één van de pioniers van de Noord Oost Polder en kregen zes kinderen. Hij had een gelukkige jeugd en was voorbestemd om de boerderij van zijn vader te continueren. Echter, de studie geneeskunde leek hem meer te passen. Na het behalen van het Havo- en het VWO- diploma in respectievelijk 1976 en 1978 deed hij zijn eerste jaar geneeskunde
aan de Universiteit van Amsterdam. Deze studie werd verder voortgezet in Groningen. Na een onderbreking van een jaar in verband met een wereldreis rondde hij de studie af in april 1986 in Groningen. Tijdens de co-schappen in Deventer was de belangstelling gegroeid voor kindergeneeskunde (Dr. J. van der Vlugt en Drs. H. Holl), wat vorm gegeven werd door een afstudeer project 'biochemische kenmerken van neuroblastoom patiënten' in het Emma Kinder Ziekenhuis in Amsterdam ( Prof. Dr. P.A. Voute en Drs. N. Abeling, hoofd biochemie). Vanaf 1986 was hij werkzaam als arts-assistent kindergeneeskunde in de Weezenlanden te Zwolle (Hoofd: Dr. F. van de Logt) en vanaf 1987 in het Wilhelmina Kinder Ziekenhuis te Utrecht (Hoofd: Prof. Dr. J.L. Van de Brande; Mentor Dr. J. Roord). In april 1988 werd begonnen met de specialistenopleiding kindergeneeskunde in het Academisch Ziekenhuis Nijmegen (Opleiders: Prof. Dr. G.B. Stoelinga en Prof. Dr. R.C. Sengers), en later het Joseph Ziekenhuis te Veldhoven (Opleider: Dr. E. Lommen). In 1991 en 1992 was hij voorzitter van de Junior Afdeling Kindergeneeskunde.
In 1993 startte hij de werkzaamheden als kinderarts in Lelystad. Sinds 2001 is hij werkzaam in het Màxima Medisch Centrum in Veldhoven. Een deeltijd opleiding in de kinderneurologie werd gevolgd in 2001 en 2002 (programma samensteller Prof. Dr. J Rotteveel). Van 2002 tot 2005 was hij voorzitter van de vakgroep Kindergeneeskunde van het Màxima Medisch Centrum. Tevens was hij vanaf 2008 co-assistent opleider kindergeneeskunde, en vanaf 2013 waarnemend arts-assistent opleider.
De werkzaamheden voor het onderzoek van dit proefschrift startten in 2006, 'deze reis lijkt nu ten einde'. Het volgende reisdoel is de toepassing van de opgedane kennis in een betere therapie van PDE, gefocust op beperking van het eiwit lysine in het dieet en optimalisering van de pyridoxine dosering.
Vinus is gelukkig getrouwd met Marita Cals. Samen zijn zij de trotse ouders van Rosalie, Benjamin en Gidius Bok.

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